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6. AUTHOR(S)

Jacqueline Biscardi, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Virginia
Charlottesville, Virginia 22906

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ABSTRACT

Interactions between HER1 and c-Src may contribute to a more aggressive phenotype in human breast cancer. Previous work from our laboratory has demonstrated that c-Src and HER1 may interact synergistically in a fibroblast model system to potentiate transformation. Here, I show that HER1 was overexpressed in 5/14 human breast cancer cell lines examined, and this overexpression correlated with an EGF-dependent physical association between HER1 and c-Src. These findings also correlated with increases in phosphorylation of the HER1 substrate Shc and activation of its downstream effector MAPK, with respect to cells that did not overexpress HER1; and with increased tumorigenicity in nude mice. In a mouse fibroblast model system, and in MDA468 breast cancer cells, HER1 in association with c-Src became phosphorylated on two novel sites: Tyr845 and Tyr1101. Phosphorylation of Tyr 845 was not observed in cells expressing kinase inactive c-Src, suggesting that this site is a target for the c-Src kinase. Tyr 845 is positioned in the activation lip of the tyrosine kinase catalytic domain, and this residue is critical to catalytic function in other kinases. In this regard, cells expressing a Y845F variant of HER1 were greatly impaired in their ability to synthesize DNA in response to both EGF and serum.

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PRINCIPAL INVESTIGATOR: Jacqueline Biscardi, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, Virginia 22906

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U.S. Army Medical Research and Materiel Command
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Jacqueline S. Brand 7/30/98

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INTRODUCTION

While a variety of effective treatments exist for early stage, noninvasive breast cancer, patients with large, metastatic tumors or recurrent tumors often fail to respond to conventional radiation and chemotherapy. Thus, investigations into molecular interactions that are specific to later stages in breast cancer progression are important in laying the groundwork for future drug design. One potential target is the human epidermal growth factor receptor (HER1/EGFR), overexpression of which correlates with a poorer prognosis [Sainsbury et al., 1985]. In addition to breast cancer, increased expression of HER1 has been shown to occur in glioblastomas and carcinomas of the prostate, bladder, kidney, stomach, ovary, and lung [Hendler et al., 1984; Hunts et al., 1985; Khazaie et al., 1983; Libermann et al., 1985; Singletary et al.,1987].

HER1 belongs to a family of receptor tyrosine kinases which includes HER2/*neu*, HER3, and HER4 [Ullrich and Schlessinger, 1990]. Amplification or overexpression of the genes encoding one or more of the HER family members is estimated to occur in approximately 67% of human breast cancers [Harris et al., 1992]. Overexpression of HER1 and/or HER2 protein occurs in 20-30% of human breast tumors, and in the case of HER1 correlates with the loss of estrogen responsiveness [Sainsbury et al., 1985; Singletary et al., 1987; Koenders et al., 1991; Slamon et al., 1986], suggesting that EGF-mediated pathways could be involved in progression to a more aggressive stage. The fact that HER2/*neu* is localized mainly in the primary tumor mass and in earlier stage *in situ* carcinomas [De Potter et al., 1988; Gusterson et al., 1988 a, b; Maguire et al., 1992; Ramachandra et al., 1990; van de Vijver et al., 1988] suggests that this molecule is involved in controlling the earlier stages of breast cancer. In contrast, HER1 has been shown to occur at higher levels in metastatic sites as compared to primary tumors [Sainsbury et al., 1987; Toi et al., 1991; Battaglia et al., 1988], and induces an invasive phenotype when expressed in non-invasive, rat mammary epithelial cell lines [Lichtner et al., 1995; Kaufmann et al., 1996]. Moreover, overexpression of HER1 protein in a murine fibroblast model system leads to transformation when cells are grown in the continuous presence of EGF, demonstrating its potential as an oncogene [Velu et al., 1987]. Taken together, these findings point to a role for HER1 in the later stages of breast cancer.

Expression and/or activity of the nonreceptor tyrosine kinase c-Src is also increased in many different human tumors, including carcinomas of the breast, stomach, ovary, colon, and prostate [Cartwright et al., 1989; Jacobs and Rubsamen, 1983; Ottenhalff-Kalff et al., 1992; Rosen et al., 1986]. In a study by Ottenhalf-Kalff et al. (1992), 100% of primary human breast tumors examined displayed increased overall tyrosine kinase activity, and 70% of the cytosolic tyrosine kinase activity was due to c-Src. Other evidence also indicates that c-Src is involved in the genesis and/or progression of human breast cancer. Earlier work from our laboratory demonstrated that overexpression of c-Src potentiates EGF-dependent DNA synthesis, and this effect, as well as normal responsiveness to EGF, is dependent on the presence of a functional c-Src kinase domain [Luttrell et al., 1988; Wilson et al., 1989]. c-Src has also been shown to physically associate with HER1 and HER2 in certain human and murine breast carcinoma cells [Luttrell et al., 1994; Muthuswamy and Muller, 1995; Stover et al., 1995] The fact that HER1 and c-Src are overexpressed in

many of the same tumor types, along with the dependence of EGF signalling on c-Src kinase, suggests that c-Src and HER1 may interact in some manner to potentiate tumorigenesis.

To investigate such a possibility, our laboratory created a panel of C3H10T½ mouse fibroblasts, which singly overexpress either c-Src or HER1, or which overexpress both these kinases. In cells overexpressing both HER1 and c-Src, a dramatic and synergistic increase in EGF-induced DNA synthesis, colony formation in soft agar, and tumor growth in nude mice was observed [Maa et al., 1995]. HER1 from the double overexpressing cells associated with c-Src in an EGF-dependent manner; and when complexed with c-Src, became phosphorylated on two previously unidentified tyrosyl residues [Maa et al., 1995]. The presence of the novel receptor phosphorylations correlated with the hyperphosphorylation on tyrosine of two HER1 substrates, Shc and PLCγ, [Pelicci et al., 1992; Rhee 1991; Ruff-Jamison et al., 1993], whose tyrosyl phosphorylations were enhanced in double overexpressing cells [Maa et al., 1995]. These results suggested that phosphorylation of HER1 on one or both of these sites could increase its kinase activity. This increased activity in turn could affect signalling through downstream targets to result in enhanced cell growth. Taken together, these findings indicate that in a fibroblast model system, c-Src and HER1 can act synergistically to promote tumorigenesis.

To assess the possibility that c-Src and HER1 cooperate in a similar fashion during the genesis of human breast cancer, a panel of breast tumor cell lines and tissue samples was examined for levels of c-Src and HER1 protein, the presence of heterocomplexes between the two tyrosine kinases, levels of tyrosyl phosphorylation on downstream signalling targets, and tumorigenesis (Specific Aim I). Rather than conduct an exhaustive survey of all known breast cancer cell lines, I chose to focus on a few representatives (14 cell lines). Some of these cell lines express receptors for both estrogen and progesterone and are thus thought to represent an earlier stage in the progression of breast cancer, while others are negative for these receptors and are thought to represent later disease stages. The results obtained from these breast cancer cell lines, which will be explained below, are consistent with the murine fibroblast model and support the hypothesis that dual overexpression of c-Src and HER1 in breast cancer cells may contribute to their tumorigenicity. These results are also the subject of a recent publication in *Molecular Carcinogenesis* [Biscardi et al., 1998].

The biochemical mechanism underlying the apparent synergy between c-Src and HER1 signalling pathways is unclear. Previous findings from our laboratory show that HER1 became phosphorylated on novel tyrosyl residues when in association with c-Src. These findings suggest that c-Src-dependent phosphorylations on the EGFR could result in hyperactivation of receptor kinase activity, as measured by the enhanced ability of the receptor to phosphorylate its cognate substrates. In this regard, I have undertaken the identification of these novel tyrosyl phosphorylations, as described in my grant proposal. I have identified Tyr 845 and Tyr 1101 as c-Src dependent sites of phosphorylation, which are present both *in vitro* and *in vivo* in c-Src associated receptor from 10T1/2 double overexpressing fibroblasts, and from MDA468 human breast cancer cells. A manuscript dealing with these issues has been submitted for publication [Biscardi et al., 1998b]. Amino acid sequences surrounding the Tyr 845 position are highly

conserved among tyrosine kinases [Hanks et al., 1988]. This tyrosine resides in the activation lip of the tyrosine kinase, and in other tyrosine kinases its phosphorylation is critical for catalytic activity [Yamaguchi and Hendrickson, 1988; Hubbard 1997; Russo et al., 1996]. Deletion of this phosphorylation by mutagenesis results in the ablation of downstream signals emanating from each particular receptor [Ellis et al., 1986; Fantl et al., 1989; van der Geer et al., 1991; Vigna et al., 1994; Mohammadi et al., 1996]. Interestingly, the HER1 homolog Tyr 845 has never before been shown to be phosphorylated. For this reason, I have chosen to focus on the biological consequences of phosphorylation at Tyr 845, with the working hypothesis that loss of phosphorylation at this position will decrease HER1-mediated signalling and cell growth. In collaboration with David Tice, a graduate student in the laboratory, I have begun investigation into the biological and biochemical role played by phosphorylation at position 845, in response to both EGF and to agonists that signal to HER1 via other types of receptors..These results will be described below, and are the subject of a collaborative paper that was recently submitted for publication [Tice et al., 1998].

MATERIALS AND METHODS

Cell lines. The derivation and characterization of the clonal C3H10T1/2 murine fibroblast cell lines used in this study, Neo (control), 5H (c-Src overexpressor), NeoR1 (human EGFR overexpressor), and 5HR11 (c-Src/EGFR double overexpressor) have been described previously [Luttrell et al., 1988; Wilson et al., 1989; Maa et al., 1995]. 5H and 5HR11 express equal levels of c-Src (~ 25 fold over endogenous), and NeoR and 5HR11 express nearly equal levels of cell surface receptors (~ 2 X 10^5 receptors/cell or ~ 40 fold over endogenous). EGFR/K- cells (overexpressing wild type HER1 and kinase deficient c-Src) were derived by infection of cells expressing kinase deficient c-Src (K- cells) with a recombinant amphotrophic retrovirus encoding HER1 [Velu et al., 1987], cloning by limiting dilution, and screening for overexpression of the receptor by Western immunoblotting. To create cells overexpressing HER1 containing a Tyr-Phe mutation at position 845 (10T845 cells), a DraIII-BstEII fragment from pCO11 (gift of Laura Beguinot), including the mutation at position 845, was subcloned into a pcDNA vector containing wild type human HER1 (gift of Dr. Stuart Decker, Parke Davis, MI). 10T845 cells were derived by Lipofectin™ (GIBCO-BRL) – mediated gene transfer and screening and selection as above. Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Gaithersburg, MD, USA), containing 10% fetal calf serum, antibiotics and G418 (400 μg/ml). When indicated, confluent cultures were starved of serum prior to stimulation with 100 ng/ml purified mouse EGF (Sigma, St. Louis, MO USA). Human breast tumor cell lines were either obtained from ATCC, or were a gift from Dr. Neal Rosen, Sloan Kettering Cancer center, New York), and were maintained in DMEM plus 5% fetal calf serum. For overexpression of c-Src in breast cancer cells, pcDNAc-Src was constructed by inserting the c-Src XhoI fragment from an existing pVZneo vector into the multicloning site of pcDNA3 (Invitrogen, San Diego, CA, USA). MDA468 cells stably overexpressing chicken c-Src (clone MDA468Src) were generated by Lipofectin™ (GIBCO-BRL) - mediated gene transfer of pcDNAc-Src into parental MDA468

cells and selection with 400 μg/ml G418. Parental MDA468 cells overexpress c-Src approximately 5 fold, as compared to Hs578Bst normal breast epithelial cells, and contain approximately 10^6 receptors/cell (19 and N. Rosen, personal communication); while MDA468Src cells overexpress c-Src approximately 25 fold over levels found in normal breast epithelial cells. 10T½ cells were maintained in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum, antibiotics and G418 (400 μg/ml). Tumor cell lines MDA468, MDA231, MCF7, and ZR75-1 were obtained from N. Rosen (Sloan Kettering, New York, USA), and the other cell lines were purchased from ATCC. Tumor cells were maintained in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum. Where indicated, confluent populations were serum-starved overnight and stimulated for the indicated times with 100 ng/ml purified mouse EGF (Sigma, St. Louis, MO, USA), 10μM lysophosphatidic acid (LPA), 2 U/mL thrombin (thr), 10 μM endothelin (ET), 10 μM isoproterenol (iso), or 500 ng/ml growth hormone (GH).

Antibodies. EGFR-specific mouse monoclonal antibodies (Mabs) 3A and 4A, were provided by D. McCarley and R. Schatzman of Syntex Research, Palo Alto, CA. Their derivation has been described previously, and their epitopes have been mapped to residues 889-944 and 1052-1134, respectively. EGFR-specific Mab F4, directed against amino acids 985-996, was obtained from Sigma (St. Louis, MO, USA). GD11 antibody is directed against the SH3 domain of c-Src and was characterized previously in our laboratory [Parsons et al., 1984; Parsons et al., 1986]. Q9 antibody, which was kindly provided by M. Payne, was raised in rabbits against the C-terminal peptide of c-Src (residues 522-533) and exhibits a higher affinity for c-Src than for other Src family members (20,21). Other c-Src antibodies used were monoclonal antibody (Mab) 2-17, directed against amino acids 2-17 (Quality Biotech, Camden, NJ, USA), MAb EC10, which is specific for the chicken form of c-Src, and Mab GD11, directed against the SH3 domain. Anti-phosphotyrosine (pTyr) polyclonal antibody (RC20) was purchased from Transduction Laboratories (Lexington, KY, USA), and Shc polyclonal antibodies were purchased from UBI (Lake Placid, NY, USA). Anti-estrogen receptor (ER) Mab, directed against residues 495-595, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-MAPK antibody was obtained from Promega (Madison, WI, USA), and anti-pan MAPK antibody (B3B9) was the gift of M. Weber [Reuter et al., 1995]. Negative control antibodies included pooled and purified normal rabbit or mouse immunoglobulin (Jackson Immunoresearch Labs, West Grove, PA, USA).

Immunoprecipitation, Western immunoblotting and *in vitro* kinase assays. Methods for immunoprecipitation, Western immunoblotting and *in vitro* kinase assays have been described previously [Luttrell et al., 1988; Wilson et al., 1989; Maa et al., 1995]. Cells were lysed either in CHAPS detergent buffer (10 mM CHAPS, 50 mM Tris HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 50 μg/ml leupeptin, and 0.5% aprotinin), or in RIPA detergent buffer (0.25% Na deoxycholate, 1% NP-40, 50 mM Tris HCl [pH 7.2], 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, and 0.5% aprotinin). Protein concentrations of detergent lysates were determined by using the BCA protein assay (Pierce, Rockford, IL, USA). 500 μg cell lysate was used for immunoprecipitations, and 50 μg was used for Western blotting. For Western immunoblotting, binding of

primary murine or rabbit antibodies to Immobilon membranes was detected with either [^{125}I]-labeled goat anti-mouse IgG (New England Nuclear [NEN], Wilmington DE, USA) or [^{125}I]-protein A (NEN) used at 1 μCi/ml, specific activity 100 μCi/ml. For kinase assays, immunoprecipitates were prepared in and washed twice in CHAPS buffer then washed twice with HBS buffer (150 mM NaCl, 20 mM HEPES, pH 7.4). Each kinase reaction was conducted in 20 μl volumes containing 20 mM PIPES, pH 7.5, 10 mM $MnCl_2$, and 10 μCi γ-[^{32}P]-ATP (6000 Ci/mmol, NEN) for 10 min at room temperature. Incubations were terminated by addition of sample buffer, and labeled products were resolved by SDS/PAGE and visualized by autoradiography.

Transient transfection and BrDU incorporation. 5H cells (overexpressing wild type c-Src) or Neo control cells were transiently transfected with 30 μg Superfect (Qiagen, Chatsworth CA) and 4 μg vector, wt HER1, or Y845F HER1 plasmid DNA according to the manufacturers' directions and incubated for 48 hr. Transfected cells were serum starved for 30 hr prior to the addition of 100 μM BrDU and either 100 ng/ml EGF or 10% fetal calf serum in growth medium, followed by incubation for an additional 18 hr and co-staining for HER1 expression and BrDU incorporation as described by the manufacturer of the BrDU-specific antibody (Boehringer Mannheim, Indianapolis IN). Fixed cells were treated with 2N HCl for 1 hr at 37 C and incubated with a mixture of primary antibodies (1:100 dilution of the HER1-specific Ab-4, and a 1:15 dilution of anti-BrDU mouse antibody). Followed by incubation with a mixture of secondary antibodies (75 μg/ml FITC-conjugated goat anti-rabbit IgG and 4 μg/ml texas red conjugated goat anti-mouse IgG, both from Jackson Immunoresearch Laboratories, West Grove PA.

Enolase assays. c-Src was immunoprecipitated from 10T½ or breast tumor cells using a pool of anti-Src Mabs, GD11 and 2-17, as described above. 2 μg acid-treated enolase was added to washed immunoprecipitates, and reactions were carried out for 10 min at room temperature, as described in Piwnica-Worms et al. [1987]. To normalize the amount of c-Src precipitated from each cell line, c-Src immunoprecipitates were immunoblotted with 2-17 antibody.

Nude mice studies. $2x10^6$ cells were injected subcutaneously into each hindquarter of 6-7 week old female nude mice (nu/nu; Charles River). For each cell line studied, 4-5 mice were injected for a total of 8-10 potential tumor sites per cell line. Tumor sizes were measured weekly with microcalipers. Tumor volume was calculated by the formula $V=LxW^2 \times 0.5$, where L is length and W is width in millimeters.

Metabolic labeling. NeoR1 or 5HR11 cells were grown to 50-75% confluency in 150 mm dishes, washed with phosphate free DMEM, and incubated for 18 hr in phosphate free DMEM containing 0.1% dialyzed fetal bovine serum and 1 mCi/ml [^{32}P]-orthophosphate (NEN) in a final volume of 10 ml. For prevanadate treatment, labeling medium was adjusted to a concentration of 3 mM H_2O_2 and 5 μM Na_3VO_4 just prior to EGF stimulation. Cells were stimulated in the presence of pervanadate by addition of 100 ng/ml EGF to the labeling medium for 5 min, washed twice with phosphate free DMEM, and lysed in CHAPS detergent buffer. Extract from an entire plate (approx. 1-2 mg protein extract) was immunoprecipitated with c-Src or EGFR specific antibodies as described above.

Two-dimensional tryptic phosphopeptide analysis. Immunoprecipitates of *in vitro* or *in vivo* [^{32}P]-labeled EGFR were resolved by SDS/PAGE. The EGFR was localized by autoradiography, excised from the gel and digested with trypsin as described by Boyle *et al.* [1991]. Phosphotryptic peptides were separated by electrophoresis at pH 1.9 in the first dimension and ascending chromatography in the second dimension on cellulose thin layer chromatography (TLC) plates. Chromatography buffer contained isobutyric acid: n-butanol: pyridine: acetic acid: H_2O (125:3.8:9.6:5.8:55.8). Migration of synthetic phosphopeptides was detected by spraying the dried TLC plate with a hypochlorite solution consisting of sequential sprays with 10% commercial Clorox, 95% ethanol, 1 % potassium iodide, and saturated o-tolidine in 1.5 M acetic acid, as described in Stewart and Young [1984].

Edman degradation. HPLC fractions of [^{32}P]-labeled EGFR phosphotryptic peptides or spots eluted from TLC plates were subjected to automated Edman degradation, as performed by the University of Virginia Biomolecular Research Facility. Briefly, phosphorylated peptides were coupled to a Sequelon aryl amine membrane [Coull et al., 1991], washed with 4 X 1 ml 27% acetonitrile, 9% TFA and 2 X 1 ml 50% methanol, and transferred to an applied Biosystems 470A sequenator using the cartridge inverted as suggested by Stokoe *et al.* [1992]. The cycle used for sequencing was based on that of Meyer *et al.* [1991], but modified by direct collection of AZT amino acids in neet TFA as described by Russo *et al.* [1992]. Radioactivity was measured by Cerenkov counting.

Identification of peptides "0" and "3". Phosphorylated peptides (corresponding to residues GMN(Y-P)LEDR, candidate for peptide "3"; or E(Y-P)HAEGGK, candidate for peptide "0") were synthesized by the University of Virginia Biomolecular Research Facility. Synthetic peptides were mixed with oxidized, *in vitro*- labeled phosphotryptic peptides from c-Src- associated EGFR, separated on cellulose TLC plates, and visualized by spraying with the hypochlorite solution as described above. One candidate for peptide "3" (GMNYLEDR) was synthesized as a phosphopeptide and tested for comigration as above. Another candidate for peptide "3" (DPHY^{1101}QDPHSTAVGNPEYLNTVQPTCVNSTF DSPAHWAQK), which was too large to chemically synthesize, was tested by further digestion of *in vitro* labeled peptide "3" with a proline-directed protease (Seikagaku, Rockville, MD, USA), according to the method of Boyle *et al.* [1991]. In brief, the spot corresponding to peptide "3" was scraped off the TLC plate, eluted with pH 1.9 buffer, and digested with 5 units proline- directed protease in 50 mM ammonium bicarbonate at pH 7.6 at 37°C for 1 hr. Peptides were separated by 2 dimensional electrophoresis as described above.

RESULTS

I. c-Src/HER1 interactions in human breast tumor cell lines.

In vivo association between c-Src and HER1 in human breast tumor cell lines.

To determine whether the c-Src/HER1 synergism model derived from the 10T½ mouse fibroblast system is relevant to human breast cancer, 14 breast tumor cell lines were first examined for their expression levels of HER1 and c-Src by immunoblotting. Figure 1 shows that five of these cell lines, SKBR3, MDA468, BT-549, MDA231, and BT-20 (lanes 4, 7, 10, 11, and 12, respectively) contained elevated levels of HER1

protein when compared to a "normal" control cell line (Hs578Bst, an immortalized, non-tumorigenic breast epithelial cell line; lane 14). Nine of the cell lines (MDA175, SKBR3, MDA361, MDA468, ZR75, BT-549, MDA231, BT-20, and MCF7; lanes 1, 4, 5, 7, 8,1 0, 11, 12, and 13, respectively) and MDA415 (Table I) exhibited increased amounts of c-Src protein. Relative levels of HER1 and c-Src were determined by densitometry, and the results are summarized in Table I. Interestingly, when the same cell lines were examined for levels of HER2, HER1 and HER2 overexpression appeared to be mutually exclusive (data not shown). The potential significance of this finding is discussed below.

To determine whether c-Src and HER1 formed heterocomplexes in the various cell lines, c-Src was immunoprecipitated with an anti-C terminal c-Src antibody from cells mock-treated or stimulated with EGF. The resulting immunoprecipitates were examined for the presence of co-precipitating HER1 by performing immune complex kinase reactions, as described previously for 10T½ cells [Maa et al., 1995]. After 1 min stimulation of the 5HR11 cells (included as a positive control) with EGF, a band of Mr 170 kDa was seen to co-precipitate with c-Src (Fig. 2A, lane 2). The intensity of this band dramatically increased after 10 min stimulation (lane 3) and was greater than the negative antibody control, also prepared after 10 min of EGF stimulation (lane 4). The 170 kDa band from lane 3 was shown to correspond to HER1 by excising it from the gel and comparing its tryptic map to that of non-c-Src-associated HER1, immunoprecipitated with anti-HER1 antibody from 10T½ cells overexpressing HER1 alone (NeoR1 cells; data not shown). In MDA468, MDA231 and BT-549 cells, which overexpressed HER1, a 170 kDa phosphoprotein co-migrating with HER1 also co-precipitated with c-Src in an EGF-dependent manner (Fig. 2B, lanes 1, 3 and 6). This protein was also confirmed to be HER1 by phosphotryptic mapping. Analysis of additional cell lines revealed that a c-Src/HER1 heterocomplex also existed in SKBR3 and BT-20 cells (Table I), but not in MCF7 or ZR75 cells (Fig. 2B, lanes 8-11), nor in any of the other remaining cell lines, regardless of whether they overexpressed c-Src. Thus, association between HER1 and c-Src was observed only in those cell lines that overexpressed both HER1 and c-Src, as in the mouse fibroblast model.

Interestingly, 4 out of the 5 cell lines positive for c-Src/HER1 interaction were also estrogen receptor (ER) negative, as revealed by immunoblotting with an antibody directed to the C-terminus of the ER (Table I). No HER1 was observed to co-precipitate with c-Src from MCF7 or ZR75 cells (Fig: 2B, lanes 8 and 10) or from BT-474, MDA361, or MDA175 cells (Table I), which overexpress c-Src but not HER1 and are positive for ER expression. While the ER status of the primary tumors from which these cell lines originated is not known, there does appear to be a correlation between ER negativity and c-Src/HER1 heterocomplex formation in the cell lines.

Immunoprecipitating c-Src with different antibodies, either a monoclonal directed to the N terminus of c-Src (amino acids 2-17) or one to the SH3 domain of c-Src (GD11) also resulted in co-precipitation of HER1 from MDA468 and MDA231 cells, but not from MCF7 or ZR75 cells (not shown). In breast tumor cell lines, numerous other bands were seen to co-precipitate with c-Src and be labeled in the kinase

reaction, but the 170 kDa band was the only one that was present in an EGF-dependent, antibody -specific manner.

To see if c-Src/HER1 heterocomplexes could also be detected in tumors, biopsy samples from primary human breast tumors were obtained from patients at the University of Virginia and analyzed as described above. Of 3 tumor samples depicted, one, from a patient with recurrent grade III adenocarcinoma (#52), exhibited elevated expression of HER1 and c-Src as revealed by immunoblotting (Fig. 3, panel A). Interestingly, c-Src immunoprecipitates from tumor #52 also showed a band co- migrating with HER1 at 170 kDa, indicating that a c-Src/HER1 heterocomplex existed in this particular tumor (Fig. 3, panel B). The presence of this complex in tumor #52 was not dependent on addition of exogenous EGF, suggesting that an autocrine mechanism, perhaps involving TGFα, may be operative in this tumor. A c-Src/HER1 heterocomplex was not observed in samples of normal breast tissue, where c-Src expression was low and HER1 expression was undetectable by Western immunoblotting, nor in the remaining two tumor samples, where the c-Src level was high but receptor expression was low.

The data from both the cell lines and biopsy samples are consistent with our earlier findings in 10T½ cells, where we demonstrated that overexpression of both c-Src and HER1 were required to observe complex formation. The only exception in our current studies is that we were unable to find an example of a human breast cancer cell line or tumor tissue which overexpressed the receptor but not c-Src. This finding suggests that overexpression of c-Src may precede overexpression of HER1 in the etiology of human breast tumors. For the remaining studies, we chose to focus on 5 representative cell lines: 3 that were "positive" for c-Src/HER1 interactions (MDA468, MDA231, and BT-549) and 2 that were "negative" for c-Src/HER1 interactions (MCF7 and ZR75).

Activity of c-Src in tumor cell lines.

The ability of c-Src to form a heterocomplex with HER1 may be dependent on the increased expression of c-Src and/or on the elevation of c-Src specific kinase activity. To determine whether c-Src kinase activity was altered, five breast tumor cell lines were assayed for the relative specific kinase activity in c-Src immunoprecipitates using acid-denatured enolase as a substrate. Figure 4 demonstrates that all the tumor cell lines examined displayed approximately equal levels of c-Src specific activity, which was not significantly different from that found in normal fibroblast controls (Neo cells) or in the double overexpressing fibroblasts (5HR11). However, Src activity was clearly elevated in the v-Src transformed IV5 cell line, which was included as a positive control. In addition, no consistent changes in c-Src kinase activity were observed when cells were stimulated with 100 ng/ml EGF for 10 minutes (not shown). Therefore, in all the cell lines examined, the ability of c-Src and HER1 to associate with one another correlated with an increase in c-Src protein rather than an increase in specific kinase activity.

Downstream effectors of HER1 in breast tumor cells.

One possible mechanism responsible for the tumorigenicity of these cell lines is enhanced signalling through the increased phosphorylation of HER1 substrate proteins. Previous work from our laboratory has demonstrated that 10T½ cells overexpressing both HER1 and c-Src show an increased extent and duration

of tyrosyl phosphorylation on the HER1 substrates, Shc and PLCγ, as compared to that seen in cells overexpressing either c-Src or HER1 alone [Foster et al., 1994]. Thus, the synergistic increase in mitogenicity and tumorigenicity observed in these double overexpressing fibroblasts correlated with enhanced signalling through Shc and/or PLCγ-mediated pathways. To determine if elevated c-Src and HER1 expression as well as heterocomplex formation in breast tumor cells correlated with increased signalling through effectors downstream of the receptor, the tyrosyl phosphorylation states of Shc and PLCγ were examined by immunoprecipitation with specifc antibodies, followed by immunoblotting with phosphotyrosine specific antibodies. No tyrosyl phosphorylation of PLCγ was found in response to EGF in any of the 5 cell lines tested (data not shown). However, Fig. 5 shows that in MDA468, MDA231, and BT-549 cells, the 46 and 52 kDa isoforms of Shc were highly phosphorylated on tyrosine in response to 10 min EGF stimulation, whereas MCF7 and ZR75 cells contained significantly lower amounts of tyrosyl phosphorylated Shc, even when the slight differences in amounts of Shc in the precipitates were taken into consideration. A time course of EGF stimulation confirmed that Shc phosphorylation in MCF7 cells was similarly low at all time points examined between 2 and 30 min (not shown). Thus, high levels of Shc tyrosyl phosphorylation correlated with overexpression of HER1 and complex formation with c-Src. Whether overexpression of HER1 or complex formation with c-Src is responsible for high levels of Shc phosphorylation cannot be deduced from this analysis, but the findings are consistent with the fibroblast model which indicated that c-Src and HER1 can cooperate to augment mitogenic signal transduction through certain downstream substrates. These results also suggest that HER1 and c-Src may signal through both PLCγ and Shc in fibroblasts, while in epithelial tumor cells, signalling through Shc or other substrates may predominate.

To investigate EGF-dependent signalling events that are further downstream, the activation state of MAPK was determined by using an antibody specific to the phosphorylated (and therefore activated) form of MAPK. Fig. 6 shows that MAPK activity was increased following stimulation with EGF only in cell lines which displayed c-Src/HER1 interactions (MDA468, MDA231, and BT-549 cells). Furthermore, MAPK activation in MDA231 cells was present even in the absence of EGF stimulation, perhaps due to activation of another component of the HER1 mediated signalling pathway independent of Shc, such as activated Ras. In comparison, MCF7 and ZR75 cells contained little or no detectable levels of activated p42 MAPK. As with Shc, MAPK activation correlates with the detection of c-Src/HER1 heterocomplexes in breast tumor cell lines overexpressing HER1, but one cannot distinguish in these cell lines whether complex formation or HER1 overexpression is responsible for the high levels of MAPK activity.

Comparative tumorigenicity of cell lines.

To determine if a correlation between c-Src/HER1 association and enhanced tumorigenesis existed, the tumorigenicity of breast cancer cell lines in nude mice was examined. Control 10T½ cells overexpressing HER1 alone (NeoR1 cells), both HER1 and c-Src (5HR11 cells), or the tumor cell lines (MDA468, MDA231, BT-549, MCF7, and ZR75) were each injected subcutaneously into the flanks of nu/nu female mice. Tumor growth was measured every week for a period of 7-8 weeks. No exogenous estrogen was

provided in order to assess the contribution of estrogen-independent pathways to tumor growth, including the EGF receptor pathway. Table II shows that in a manner similar to that seen in our earlier studies, all mice injected with 5HR11 cells developed large, rapidly growing tumors which reached a size of 290 mm^3 in volume within 28 days of injection. In contrast to this, by day 50, only 33% of the mice injected with NeoR1 cells developed tumors, and each tumor was very small, measuring less than 1 mm in diameter. Previous studies have also shown that cells overexpressing c-Src alone fail to develop tumors greater than 1 mm in diameter [Foster et al., 1994]. Thus, in 10T½ cells, overexpression of c-Src in a background of high HER1 expression resulted in a faster growth rate and more aggressive phenotype as compared to cells overexpressing c-Src or HER1 alone.

Nude mice injected with MDA468, MDA231, and ZR75 cells also developed tumors which, after 50 days, ranged from 10 to 16 cubic millimeters in volume. A relatively small number of cells ($2x10^6$ cells/injection site) was used, so that rates of tumor growth could be more accurately measured. Only 22% of the mice injected with MCF7 cells formed tumors, which were 1 mm^3 in volume. The tumorigenic response of the BT-549 cells was also weak, which was unexpected, since their EGF-dependent signal transduction pathways behaved in a manner similar to those of MDA468 and MDA231 cells. With respect to ZR75-1 cells, only 67% of the sites injected developed tumors (compared to 100% and 90% for MDA231 and MDA468 cells, respectively), and the initial rate of growth of the ZR75-1 induced tumors was retarded with respect to the MDA468 and MDA231 induced tumors. Thus, these data suggest that c-Src/HER1 mediated signalling pathways could contribute to the tumorigenicity of MDA468 and MDA231 cells, while different mechanisms may be dominant in the MCF7, ZR75-1, and BT-549 cells.

II. Identification of c-Src-mediated phosphorylations on HER1.

***In vivo* and *in vitro* phosphorylation of novel, non-autophosphorylation sites on HER1 in complex with c-Src.**

As described above, overexpression of both HER1 and c-Src in 10T1/2 cells results in the formation of a heterocomplex between these two kinases in a number of breast cancer cell lines, as well as increased tyrosyl phosphorylation of the receptor substrate Shc following EGF treatment [Maa et al., 1995; Biscardi et al., 1998]. These findings suggest that the c-Src- associated receptor is modified in some manner as to increase its kinase activity. I have used the 10T1/2 mouse fibroblast model system in the following experiments in order to facilitate interpretation of experimental results. To examine the receptor for novel phosphorylations, the *in vitro* phosphorylated c-Src associated 170 kDa protein from 5HR cells was excised from the gel and subjected to 2-dimensional phosphotryptic peptide analysis. The phosphopeptide map of c-Src- associated receptor was then compared to the map of the free receptor, immunoprecipitated with receptor antibody. Fig. 7, Panels A and B demonstrate that the maps are nearly identical, confirming that the 170 kDa protein associated with c-Src was indeed the receptor. However, two additional phosphorylations (designated peptides "0" and "3") were seen in the map of the HER1 complexed with c-Src, suggesting that c-Src was responsible for their phosphorylation. Consistent with this notion, 2-

dimensional phosphoamino acid analysis of the *in vitro* labeled HER1 demonstrated that peptides "0" and "3" contained only phosphotyrosine (data not shown). Panel C shows that the two novel phosphopeptides could also be detected in the receptor found in complex with c-Src from ^{32}P- metabolically labeled 5HR cells that had been treated with pervanadate and EGF for 5 min. These data indicate that two c-Src-dependent phopshorylations occur on HER1 both *in vitro* and *in vivo* when c-Src becomes physically associated with the receptor following EGF stimulation.

Initial attempts to detect peptides "0" and 3" from metabolically labeled cells yielded phosphopeptide maps that contained only peptide "3". In these experiments, lysates were prepared in CHAPS buffer containing a cocktail of conventional protease and phosphatase inhibitors (see Methods). However, modification of the EGF treatment regimen to include pervanadate allowed us to detect peptide "0" in receptor immunoprecipitates from both NeoR (Panel B) and 5HR (Panel D) cells. More peptide "0" was seen in receptor from 5HR than from NeoR cells. Of special note was the finding that peptide "0" was the only peptide seen to increase in phosphorylation in response to pervanadate treatment, suggesting that its phosphorylation is indeed more labile than that of peptide "3" and the other phosphorylations on the receptor, which correspond to autophosphorylation sites. These results suggest that (1) peptide "0" is a candidate for phosphorylation by the receptor (or another receptor- regulated tyrosine kinase) as well as by c-Src, or that (2) c-Src increases receptor (or another tyrosine kinase) phosphorylation of peptide "0". Subsequent experiments (see below) suggested that the latter could be the preferred mechanism. Fig. 8, Panel A shows that in the absence of pervanadate, peptide "3" could be detected in the receptor immunoprecipitated from NeoR cells. However, phosphorylation of this peptide was consistently found to be higher in 5HR cells (Panel C). This finding was corroborated by data obtained from an HPLC profile (see below) of *in vitro* labeled, free receptor, where phosphorylation of the peak corresponding to peptide "3" increased ~3.5 fold when the receptor was associated with c-Src (data not shown).

To identify the amino acids phosphorylated in a c-Src- dependent manner, fractions containing peptides "0" and "3" were isolated by HPLC (not shown). Peptide "0" eluted at 8.5% acetonitrile, a concentration very close to that at which the previously described *in vivo*-labeled peptide SPY1 eluted [Wasilenko et al., 1991] and peptide "3" eluted at 10.5% acetonitrile (not shown). These HPLC fractions, which were of greater than 95% purity,were subjected to sequential Edman degradation to determine the cycle number at which radioactivity was released. Results from these analyses indicated that a phosphoamino acid residue was located at the second position of peptide "0" (Figure 9, Panel A) and at the fourth position of peptide "3" (Figure 9, Panel B). Of the tryptic peptides generated from the intracellular domain of HER1 which contain Tyr residues, those peptides containing Tyr 845, 867 or 891 were potential candidates for peptide "0", while those peptides containing Tyr 803 or 1101 were potential candidates for peptide "3".

The Tyr 845-containing peptide was selected for further study as a candidate for peptide "0", since it showed 50% homology to sequences contained within the autophosphorylation site of c-Src (Tyr 416), indicating that it could be a potential c-Src target. The octamer composed of E(P-Y^{845})HAEGGK (peptide "0") was chemically synthesized to include a phosphorylated Tyr 845 and analyzed either alone (Figure 10,

Panel A) or in a mix with total peptides from *in vitro* labeled, c-Src- associated receptor by 2-dimensional TLC (Panel C). The synthetic octamer comigrated with peptide "0" in the mix, thereby identifying Tyr 845 as the phosphorylated residue in peptide "0".

Since peptides "0" and "3" migrated similarly in the 2-D chromatography, it was expected that they would share similar isoelectric points and hydrophobicities. Both the candidate peptides for peptide "3" (GMNY^{803}LEDR or DPHY^{1101}QDPHSTAVGNPEYLNTVQPTCVNSTF DSPAHWAQK) had theoretical isoelectric points and calculated hydrophobic indices [Boyle et al., 1991] similar to those of the Tyr 845 -containing peptide, indicating that both were potential candidates. The Tyr 803- containing peptide was selected first for further study, since it was smaller and more easily synthesized. However, this synthetic phosphopeptide did not co-migrate with peptide "3" nor with any of the other HER1 phosphopeptides (data not shown), indicating that the Tyr 1101 containing peptide was the preferred candidate. To verify the identity of peptide "3", the *in vitro*- labeled peptide "3" was scraped off the TLC plate, eluted with pH 1.9 buffer, and subjected to further digestion with a proline-directed protease as described in Methods. Since, of the two candidate peptides, only the Tyr 1101-containing peptide contains proline residues, any change in mobility resulting from digestion with this protease would confirm its identity as peptide "3". As a control, peptide "0", which does not contain any proline residues, was digested with proline-directed protease and no change in mobility was observed (data not shown). Fig. 11 shows that digestion of spot "3" with the proline-directed protease resulted in a change of migration primarily in the first dimension (compare Panel A with Panel B). To confirm that a mobility shift was indeed occurring, digested and undigested peptide "3" were mixed (Panel C). Therefore, peptide "3" is identified as Tyr 1101, which. along with Tyr 845, represent novel, c-Src dependent sites of *in vivo* tyrosyl phosphorylation on the EGFR.

Phosphorylation of Tyr 845 and Tyr 1101 in HER1 from breast tumor cells.

Our laboratory has previously demonstrated the presence of EGF- dependent c-Src/HER1 heterocomplexes in several human breast tumor cell lines including MDA468, which overexpresses both c-Src and HER1 [Biscardi et al., 1998]. Since the presence of this heterocomplex is correlated with general increases in downstream receptor- mediated signaling and tumorigenicity in these cells, as compared to cell lines which do not overexpress the EGFR, we wished to investigate whether Tyr 845 and/or Tyr 1101 were phosphorylated in c-Src- associated HER1 derived from breast tumor cells. MDA468 cells overexpress EGFR at a level ~40 fold over normal breast epithelial cells (approximately 10^6 receptors/cell; N. Rosen, personal communication), while c-Src is moderately overexpressed, approximately five times greater than in normal breast epithelia. Fig. 12, Panel A demonstrates that phosphopeptides "0" and "3" are both present in *in vitro*- labeled, c-Src- associated HER1 from EGF-stimulated MDA468 cells, although peptide "0" is weakly detected. To further investigate the role of c-Src in mediating the phosphorylation of these sites, an MDA468 derivative cell line which stably overexpressed c-Src approximately 25 fold over levels in normal breast epithelial cells (MDA468c-Src cells, panel B) was created. In these cells, the phosphorylation of peptide "0" (Tyr 845) was greatly enhanced, while the phosphorylation of peptide "3" (Tyr 1101) was

unchanged (Panel C). In prelimimary studies, MDA468 cells overexpressing a kinase-deficient form of c-Src were created using techniques described above. While c-Src and HER1 physically associated, similar to the results from the 10T1/2 cell lines, no phosphorylation of Tyr 845 was observed. This result suggests that c-Src directly phosphorylates HER1 at this position in MDA468 cells as well as in the mouse fibroblast model system, as described below. Furthermore, MDA468Src and parental MDA468 cells were injected into nude mice, and the size of the resulting tumors compared. MDA468Src cells produced tumors which were approximately the same size as those produced by parental cells. Since MDA468 cells are already very aggressive, it may be impossible to increase their tumorigenicity further. For this reason, the experiments outlined in Specific Aim III of my proposal, which will use dominant negative forms and/or domains of the receptor in an effort to block tumorigenic signalling, will best address this issue.

To see if overexpression of c-Src in these breast tumor cells enhanced signaling downstream from the EGFR, as is seen in the 10T1/2 system, the levels of tyrosyl phosphorylation of the HER1 substrates Shc and PLCγ were examined. MDA468 parental or MDA468c-Src cells were stimulated with 100ng/ml EGF for the indicated times, and extracts were subjected to immunoprecipitations with Shc antibody followed by immunoblotting with either pTyr antibodies (panel C) or Shc antibodies (panel D). Tyrosyl phosphorylation of Shc derived from MDA468 cells overexpressing c-Src is increased in both extent and duration with respect to Shc derived from parental MDA468 cells (compare lanes 2-4 to lanes 6-8). Thus, increased levels of c-Src in these breast cancer cells correlates with increases in signaling through the EGFR, perhaps via the c-Src- mediated phosphorylation of Tyr 845.

III. Physiological relevance of Tyr 845 phosphorylation.

The experiments in Section III were performed in collaboration with David Tice, a graduate student in the laboratory. Phosphotryptic mapping was employed to investigate the status of Tyr 845 phosphorylation in cells which overexpressed wild type HER1 and kinase defective c-Src (EGFR/K- cells). While HER1 physically associated with c-Src in these cells in an EGF-dependent manner, phosphotryptic mapping revealed that Tyr 845 was not phosphorylated (Fig. 13), both *in vitro* (panels B, C, and D) and *in vivo* (panels E and F). Loss of Tyr 845 phosphorylation was observed in two independent clones of EGFR/K- cells (panels C and D). The lack of Tyr 845 phosphorylation seen in EGFR/K- cells correlated with the inability of these cells to form tumors in nude mice, in comparison to the large tumors formed by cells expressing wild type c-Src [Tice et al., submitted.] Furthermore, phosphorylation of Tyr 1101, the other potentially c-Src-mediated site, was also decreased, although to a lesser extent. These results suggest that Tyr 845 and, perhaps, Tyr 1101, are specific targets for c-Src; or else are phosphorylated by a secondary tyrosine, the activation of which is dependent on c-Src kinase activity. Interestingly, the other phosphorylations on the receptor were not affected by the lack of c-Src kinase activity, suggesting that c-Src specifically targets Tyr 845 and 1101.

A tyrosyl residue homologous to HER1 Tyr 845 is present in many other receptor and nonreceptor tyrosine kinases [Hanks et al., 1988]. Mutagenesis studies in other tyrosine kinases proves the importance of this phosphorylation, in that Tyr to Phe mutations at this position decrease signalling through the

receptor [Ellis et al., 1986; Fantl et al., 1989; van der Geer et al., 1991; Vigna et al., 1994]. Thus, it is possible that mutation of Tyr 845 to phenylalanine would likewise decrease EGF-dependent signalling through HER1. To directly test the requirement of Tyr 845 phosphorylation for receptor function, a variant receptor bearing a Y845F mutation was transiently transfected into 5H cells (overexpressing wild type c-Src), and the effects on mitogenesis assayed by measuring BrDU incorporation in response to both EGF and serum (Fig. 14). The level of bromodeoxyuridine (BrDU) incorporation in cells expressing the Y845F mutant HER1 was reduced to that of serum-starved cells, suggesting that phosphorylation of Tyr 845 is necessary for mitogenic function of the receptor. Interestingly, both EGF- and serum- induced BrDU incorporation were affected by the Y845F mutation. This finding is intriguing in light of recent evidence which describes cross-talk between HER1-mediated pathways and pathways mediated by G-protein coupled receptors (GPCRs). Since LPA, the active ingredient in serum, is a ligand for a GPCR, it is possible that phosphorylation of Tyr 845 is a more general phenomenon and can be elicited by other GPCR ligands. This possibility is discussed in section IV.

Despite the intriguing biological consequences of Tyr 845 phosphorylation (decreased mitogenesis and tumorigenesis), we have been unable to correlate these phenomena with a biochemical mechanism. Both Mr. Tice and myself have examined the phosphorylation and/or activation of a number of substrates downstream of HER1 (including Shc, MAPK, STAT3, PLCγ) in both the EGFR/K- cells and the 10T845 cells, and have found no differences. Furthermore, the catalytic activity of the Y845F mutant HER1 does not appear to be affected, as least as measured by an *in vitro* kinase reaction. We will continue to examine these cell lines for any biochemical changes, specifically looking at other STATs, the PI3-kinase pathway, and heterodimerization with other HER family members. Also, the 10T845 cells will be studied in soft agar growth assays and nude mice tumorigenesis assays. The phosphotryptic mapping pattern of these cell lines will also be investigated, as it is possible that the phosphorylation of other sites may be increased in a compensatory mechanism, resulting in the seemingly normal biochemical function of the Y845F receptor.

IV. Tyr 845 phosphorylation in response to GPCR pathways.

Recent studies have demonstrated that agonists traditioanlly thought to signal only through GPCR or cytokine receptor pathways actually elicit responses from HER1-mediated pathways. Lefkowitz and colleagues [Luttrell et al., 1996; Luttrell et al., 1997] have shown that in COS cells in response to LPA, the active ingredient in serum and a GPCR agonist, HER1 becomes phosphorylated and MAPK activity, the traditional downstream effector of HER1, is increased. These effects appear to be mediated by c-Src, as expression of a kinase inactive form of c-Src or the presence of CSK (cellular src kinase), which decreases c-Src kinase activity abrogates these responses. Similar work by Daub et al. [Daub et al., 1996; Daub et al., 1997] shows that LPA and endothelin both trigger HER1 phosphorylation and activation of downstream pathways. Furthermore, recent studies have shown that HER1 is phosphorylated by the JAK2 kinase in response to growth hormone (GH), which signals through a cytokine receptor [Yamauchi et al., 1997].

Taken together, these findings indicate that HER1 can mediate signals originating from a variety of extracellular agonists.

Our work with cells expressing the Y845F mutant form of HER1 showed that they were impaired in their ability to synthesize DNA in response to both EGF and serum. This finding suggests that the c-Src mediated phosphorylation of Tyr 845 may be a more general response, and occur in response to activation of signalling pathways controlled by other types of receptor. To test this possibility, 5HR cells were treated with various agonists: EGF (100 ng/ml), endothelin (ET,), growth hormone (GH,) isoproterenol (iso, 10 μM), LPA (10 μM) or thrombin (thr) for 10 min, HER1 was immunoprecipitated, and immunoblotted with phosphotyrosine specific antibodies. Of these compounds, all but iso resulted in increases in HER1 phosphorylation with respect to nontreated cells (Fig. 15, panel A). To determine if these compounds caused association between c-Src and HER1, 5HR cells or EGFR/K- cells were treated with agonists and c-Src immunoprecipitation/kinase reactions were carried out as described above. Fig. 15, panel B shows that EGF, GH, and LPA caused the greatest degree of association between c-Src and HER1, as visualized by an immune complex kinase assay. ET, iso and thr also resulted in a low degree of association that was not greatly increased over basal levels.

To determine if Tyr 845 was phosphorylated in response to these agonists, phosphotryptic peptide mapping was performed on c-Src associated HER1 derived from 5HR or EGFR/K- cells stimulated with the above agonists. Fig. 16 demonstrates that LPA, GH and endothelin were all able to induce phosphorylation at this position (compare panel A, EGF treated, with panels B, C and D). Phosphorylation of Tyr845 was not observed in free receptor derived from NeoR cells in response to these treatments (data not shown), indicating that association with c-Src is necessary for this phosphorylation to occur. Interestingly, the other c-Src-mediated phosphorylation site, Tyr 1101, was not greatly increased by treatment with agonists other than EGF, suggesting that phosphorylation at this site is a specific response to EGF.

To further investigate the role of c-Src in mediating Tyr 845 phosphorylation in response to ET, GH and LPA, phosphotryptic mapping of c-Src associated HER1 in EGFR/K- cells was performed. In these cells, phosphorylation at Tyr 845 is not observed in response to ET or GH (Fig. 16, panels E and F). The ability of other agonists to induce Y845 phosphorylation in this cell line is currently being tested. While preliminary, these findings suggest that the presence of catalytically active c-Src is critical for phosphorylation of Tyr 845, both in response to EGF and to other ligands that bind GPCRs or cytokine receptors.

DISCUSSION

I. c-Src/HER1 interactions in human breast tumor cell lines.

The studies undertaken to date provide evidence for the hypothesis that interactions between c-Src and HER1 result in synergistic increases in mitogenicity and tumorigenicity in fibroblasts as well as in human

breast cancer cell lines. In a panel of 14 breast cancer cell lines examined, the ability to detect a heterocomplex between HER1 and c-Src correlated with overexpression of both HER1 and c-Src kinase. Presence of this heterocomplex was EGF-dependent, inversely correlated with ER expression, and directly correlated with higher levels of Shc and MAPK activation and/or phosphorylation. While EGF-induced activation of c-Src was not evident in these cell lines, the increases in Shc and MAPK phosphorylation suggest that hyperactivation of the receptor could be a functional consequence of the interaction between c-Src and HER1, as was demonstrated previously in the fibroblast model system [Maa et al., 1995]. Two out of three of the cell lines exhibiting the c-Src/HER1 heterocomplex in this sampling resulted in slightly larger, faster growing, and more numerous tumors in nude mice, as compared to cell lines which did not overexpress HER1 or show c-Src/HER1 association. Thus, enhanced tumor growth could be mediated in part by a signalling pathway involving HER1, c-Src, Shc and MAPK.

Results from other laboratories also provide evidence for the hypothesis that c-Src and HER family members can form heterocomplexes in various tumor cells. Luttrell et al. [1994] demonstrated that in MDA468 cells, c-Src co-precipitates a 170 kDa phosphotyrosine containing protein, while Sato et al. [1995] showed that c-Src and HER1 associate in HER1-overexpressing A431 cells in an EGF-dependent manner. c-Src and the related family member, HER2, co-precipitate in mammary carcinoma cells derived from HER2/*neu* transgenic mice [Muthuswamy and Muller, 1995] and the isolated c-Src SH2 domain can bind HER2 immunoprecipitated from SKBR3 breast tumor cells [Luttrell et al., 1994]. While the experiments in this manuscript do not address the question of whether c-Src binds HER1 directly, other work from our laboratory indicates that these two molecules can interact directly *in vitro* in a Far Western assay [Biscardi et al., 1998b], and Muthuswamy and Muller [1995] have demonstrated direct binding between c-Src and HER2. Other data from our laboratory indicate that c-Src and HER2 also form a heterocomplex in MDA361 cells, which express high levels of HER2 (A. Belsches and S. Parsons, unpublished data). No HER1/c-Src interactions have been detected in this cell line. Interestingly, of the 14 breast tumor cell lines examined in the present study, overexpression of HER1 and HER2 appeared to be mutually exclusive, suggesting that within any one cell type c-Src binds the most abundantly expressed HER family member.

In contrast to our findings, Stover et al. [1995] found that c-Src and HER1 can associate in MCF7 cells, despite the extremely low amounts of HER1 present in these cells. We have been unable to detect HER1 in MCF7 cells, either by immunoblotting or immunoprecipitating with HER1-specific antibodies (unpublished results). While immunoprecipitations with a c-Src antibody directed against the unique domain (2-17) did occasionally result in a co-precipitating 170 kDa band in these cells, we found that this result was not reproducible. Thus, such a heterocomplex may occur in MCF7 cells but be difficult to detect, due to the low amount of receptor expressed. Alternatively, the different results could be due to heterogeneity in the MCF7 cell line itself.

The high levels of tyrosyl phosphorylation of the HER1 substrate Shc observed in those breast cancer cells where HER1 is overexpressed and associated with c-Src could be the result of several different

mechanisms: (1) elevated HER1 expression alone is responsible for the phosphorylation; (2) c-Src phosphorylates Shc itself; (3) c-Src in some way activates HER1 tyrosine kinase activity; or (4) another unidentified tyrosine kinase is activated by HER1 and c-Src and subsequently phosphorylates Shc. While the elevated Shc phosphorylation observed could be due solely to the high levels of HER1 expressed in these cells, two pieces of evidence suggest that alternative mechanisms may exist. First, in the 10T½ system, levels of Shc tyrosine phosphorylation are much higher in the double overexpressing 5HR11 fibroblasts than in NeoR1 cells, which overexpress HER1 alone [Foster et al., 1994]. Second, we have found that overexpression of c-Src in MDA468 cells results in a further EGF-dependent increase in Shc tyrosyl phosphorylation, over and above that seen in the parental MDA468 cells [Biscardi et al., 1998b]. Thus, the increased Shc phosphorylation observed in these breast cancer cells coincides with overexpression of HER1 and c-Src and the presence of heterocomplexes between these two molecules.

Another possible mechanism to explain the increased signalling through Shc and the increased tumorigenesis in MDA468 and MDA231 cells is the activation of c-Src following EGF stimulation and the subsequent phosphorylation of Shc by c-Src. Other investigators [Osherov and Levitzki, 1994; Oude Weernink et al., 1994] have reported that in cells overexpressing HER1, EGF treatment induces an increase in c-Src kinase activity. Further, Osherov and Levitzki [1994] demonstrate that in cells which overexpress HER1 at extremely high levels, such as A431 carcinoma cells, c-Src is constitutively activated independently of EGF stimulation. However, we have been unable to observe reproducibly an EGF-dependent or independent activation of c-Src in either the 10T½ system or in breast tumor cell lines. This finding is similar to the results of Campbell et al. [1996], who also failed to detect an increase in c-Src specific activity in ZR75 or MCF7 cells. Furthermore, in the 10T½ system, cells overexpressing c-Src alone exhibit reduced levels of Shc phosphorylation following EGF stimulation, as compared to cells overexpressing the receptor alone or both the receptor and c-Src. These findings suggest that c-Src alone is not responsible for enhanced Shc phosphorylation, but instead may act in concert with HER1.

Finally, the hypothesis that c-Src activates HER1 is supported by findings from the 10T½ system, where phosphorylation of Tyr 845 in c-Src-associated HER1 correlates with enhanced phosphorylation of receptor substrates [Maa et al., 1995; Biscardi et al., 1998b]. Sequences surrounding this tyrosyl residue bear 50% homology to those surrounding Tyr 416 of c-Src, phosphorylation of which is known to result in increased Src kinase activity [Cooper et al., 1993]. Another novel phosphorylation (Tyr 1101) of the receptor has been detected in kinase reactions of c-Src-associated HER1 derived from 5HR11 murine fibroblasts *in vitro* and *in vivo*, and from MDA468 or 231 cells *in vitro* [Biscardi et al., 1998b]. Therefore, the elevated levels of Shc tyrosyl phosphorylation could result from activation of HER1 by phosphorylation at either Tyr 845 or 1101, or be the result of a more complicated signalling pathway involving heterodimers with other HER family members or with other tyrosine kinases.

The finding that c-Src and HER1 form a heterocomplex in certain breast tumor cell lines suggests that interactions between these two kinases could contribute to a more aggressive tumor phenotype. Indeed, in the panel of cell lines we tested, a general correlation was found to exist between the presence of increased

tumorigenicity in nude mice and HER1/c-Src heterocomplexes. An exception was found in the BT-549 cells, which yielded very small tumors despite being positive for c-Src/HER1 interactions. Such a result is not unexpected, given the genetic heterogeneity of tumor cells in general and the multitude of signalling pathways that can contribute to tumorigenicity. Therefore, taken together with results from the $10T_{1/2}$ system, where overexpression of c-Src and HER1 results in a synergistic increase in tumor size, these data are consistent with the notion that c-Src and HER1 are critical regulators of the growth of some, but not all, human breast tumors.

HER1/HER2 overexpression occurs in 20-30 % of human breast tumors and is correlated with poorer prognosis and loss of estrogen responsiveness [Sainsbury et al., 1987; Singletary et al., 1987; Koenders et al., 1991; Slamon et al., 1986; Slamon et al., 1987; Toi et al., 1991] while c-Src expression and activity is elevated in a majority of breast tumors regardless of stage [Ottenhalff-Kalff, Rijksen 1992] {Rosen, Bolen, et al. 1986 ID: 228}. In this regard, four of five cell lines which exhibit c-Src/HER1 interactions, are ER negative (Table I), whereas six of nine cell lines which express very little HER1 and do not associate with c-Src are ER positive. While it is possible that some of the ER positive cell lines may possess non-functional receptors, our results still suggest that the presence of c-Src/HER1 complexes may be one indicator of a more aggressive phenotype.

The fact that HER1 and c-Src physically and functionally interact has potentially important therapeutic implications. Neither c-Src nor HER1 is overexpressed in normal breast tissues suggesting that complex formation and the resulting synergistic signalling occurs to a much greater extent in tumor cells than it does in normal cells. It is possible that interdiction of the signalling events enhanced by c-Src/HER1 interactions could result in decreased cell growth. Reagents that could disrupt the c-Src/HER1 heterocomplex, or otherwise interfere with the synergistic signalling between these two molecules, could be used therapeutically in a tumor-specific manner. By interdicting an event more specific to tumor cells than to normal cells, the potential side effects and toxicity that are associated with more general tyrosine kinase inhibitors could be avoided. Thus, studies such as these may provide a basis for the design of novel therapies for use in breast cancer patients.

II-IV. Identification of Tyr 845 and 1101 as c-Src mediated phosphorylation sites on HER1, and functional consequences.

Previous studies from our laboratory proved that double overexpressing 10T1/2 fibroblasts show synergistic increases in EGF-dependent mitogenesis, transformation and tumorigenesis, as well as increases in tyrosyl phosphorylation of the receptor substrates Shc and PLCγ. Moreover, in both 10T1/2 fibroblasts and breast cancer cells that overexpressed both c-Src and HER1, HER1 physically associated with c-Src in an EGF- dependent manner, and became phosphorylated on two novel sites [Maa et al., 1995]. Identification of these two novel c-Src-dependent phosphorylations is important, as they represent possible sites for therapeutic intervention. In earlier studies investigating Src's ability to potentiate growth factor receptor driven pathways, Wasilenko *et al.* [1991] demonstrated that cells co-expressing v-Src and HER1

exhibit elevated receptor tyrosine phosphorylation and kinase activity in the absence of EGF. In addition, they showed that the receptor contains two novel sites of tyrosine phosphorylation, both of which are dependent upon the presence of v-Src, and one of which (SPY 1) is identical to peptide "0" seen here and in Maa *et al.*, [1995] in the c-Src associated HER1 [Wasilenko et al., 1991]. These findings suggest that in normal, nontransformed cells HER1 may be phosphorylated and perhaps activated by c-Src but in an EGF-dependent manner. Here we have identified these sites as containing Tyr 845 and Tyr 1101, and give evidence for their dependency on c-Src and for the involvement of Tyr 845 phosphorylation in upregulating the kinase activity of HER1 in both murine fibroblasts and breast cancer cells.

Tyr 845 is in an intriguing position on the receptor, as it resides in the activation lip of the kinase domain [Yamaguchi and Hendrickson, 1996; Hubbard, 1997; Russo et al., 1996]. Phosphorylation of the analogous tyrosine in other kinases results in stabilization of the activation lip allowing access to substrate, and thus is necessary for activation of catalytic activity [Hubbard, 1997]. Sato *et al.* [1995] showed that in A431 cells a CNBr fragment containing Tyr 845 from the c-Src-associated HER1 is phosphorylated *in vitro* and *in vivo.* However, confirmation of the phosphorylation of Tyr 845 and studies detailing the signaling implications of this phosphorylation have been lacking. The fact that Tyr 845 was the only phosphorylation that was significantly enhanced following treatment of the cells with a tyrosine phosphatase inhibitor suggests that this phosphorylation is a labile event, and is tightly regulated by an as yet unidentified tyrosine phosphatase. For this reason, its presence has been difficult to detect. Mutation of these analogous sites in their respective receptors has been shown to cause decreases in cell growth and transformation. [Ellis et al., 1986; Fantl et al., 1989; van der Geer et al., 1991; Vigna et al., 1994; Mohammadi et al., 1996]. Interestingly, a tyrosine at this position is not found in HER1 family member erbB3/HER3, which is known to lack kinase activity [Guy et al., 1994]. Thus, phosphorylation of this tyrosyl residue may regulate catalytic activity.

Whether phosphorylation of Tyr 845 and/or Tyr 1101 in cells overexpressing c-Src results in hyperactivation of the receptor remains an open question. Phosphorylation of Tyr 845 and 1101 in 5HR cells correlates with the increased tyrosyl phosphorylation of HER1 substrates Shc and PLCγ [Maa et al., 1995]. Taken together with the enhanced mitogenicity and tumorigenicity in these double overexpressing cells, these results suggest that phosphorylation of the receptor on Tyr 845 and/or 1101 may hyperactivate receptor kinase activity. Overexpression of c-Src, as seen in our 10T1/2 system or as occurs naturally during the course of oncogenesis in a breast tumor cell, appears to result in the enhanced phosphorylation of Tyr 845. Moreover, c-Src kinase activity is absolutely required for phosphorylation of Tyr 845 to occur, as no phsoshorylation at this position is seen in cells expressing kinase deficient c-Src. This finding argues for the possibility that c-Src phosphorylates Tyr 845 directly, or else activates a secondary tyrosine kinase to result in phosphorylation at this position. In cells that do not contain elevated levels of c-Src, it is possible that Tyr 845 becomes phosphorylated on a subset of HER1 molecules, but the stoichiometry and labile nature of this phosphorylation make it impossible to detect. Overexpression of c-Src may result in

the activation of a greater number of HER1 molecules, so that Tyr 845 phosphorylation becomes detectable.

It is possible that c-Src, rather than the receptor, is responsible for the increased phosphorylation of PLCγ and Shc previously observed in the 10T1/2 system. In previous work from other laboratories, EGF stimulation of cells overexpressing HER1 resulted in increased kinase activity of c-Src [Osherov and Levitzski, 1994; Oude Weernink et al.,1994]. However, we did not consistently observe this phenomenon observed in the 10T1/2 system or in a panel of breast cancer cell lines [Maa et al., 1995; Biscardi et al., 1998]. In this regard, while v-Src is capable of phosphorylating PLCγ *in vitro* and Shc *in vitro* and *in vivo* [Oude Weernink et al., 1994], c-Src overexpression alone in 10T1/2 cells does not result in hyperphosphorylation of either PLCγ or Shc in response to EGF [Maa et al., 1995]. Thus, it is possible that the receptor becomes hyperactivated upon phosphorylation of Tyr 845, and, possibly, Tyr 1101. Since hyperphosphorylation of HER1 substrates is seen only in the double overexpressing cells, we propose that c-Src and HER1 may act together synergistically to induce the enhanced signaling observed.

A potential role for Tyr 1101 is not as clear, as this residue is not conserved among HER family members. This could indicate that an EGF-specific signalling molecule interacts with this site, perhaps in an SH2-dependent manner similar to that of the autophosphorylation sites in the C terminus. Conversely, heterodimerization could result in the phosphorylation of Tyr 1101, which in turn could be the site of interaction with c-Src. Previous work has shown that the SH2 domain of c-Src can bind peptides containing phosphorylated Tyr 992 [Luttrell et al., 1994; Sierke et al., 1993] and Tyr 1101 *in vitro* [Lombardo et al., 1995]. Thus, c-Src could bind one of these sites, which could position it to phosphorylate Tyr 845. Lombardo et al. [1995] demonstrated that Tyr 1101 of HER1 is a site of *in vitro* phosphorylation by c-Src. Here, we show that this phosphorylation also occurs *in vivo*. In other studies, Stover et al. [1995] showed that Tyr 891 and Tyr 920 were phosphorylated in the c-Src-associated HER1 derived from MCF7 cells. However, we did not detect these phosphorylations in either the 10T1/2 or the MDA468 cells, and were unable to detect any HER1 in MCF7 cells by Western blotting [Biscardi et al., 1998].

The ability of c-Src to phosphorylate a site on HER1 that bears homology with its own autophosphorylation site strengthens the notion that HER1 is phosphorylated by c-Src directly. Further evidence comes from our studies with MDA468 cells overexpressing c-Src, where an enhanced phosphorylation of Tyr 845 is observed. Moreover, we have demonstrated that overexpression of a kinase inactive form of c-Src in 10T1/2 cells overexpressing HER1 results in a decrease in Tyr 845 phosphorylation [Tice *et al.,* 1998]. This finding indicates that c-Src kinase activity is necessary for the phosphorylation of Tyr 845, either by directly phosphorylating this site, or by activating another tyrosine kinase. *In vitro* affinity precipitation and Far Western analysis demonstrate that the c-Src SH2 domain can bind activated HER1 specifically and directly [Biscardi et al., 1998b; Lombardo et al., 1995; Luttrell et al., 1994; Sierke et al., 1993]. However, other HER family members (including HER2) [Carraway and Cantley, 1994; Qian et al., 1992; Dougall et al., 1993] and several cytosolic tyrosine kinases, such as other

c-Src family members and JAK kinases have been reported to be involved in receptor-mediated signalling; and we cannot completely exclude their possible involvement in phosphorylation of Tyr 845 and Tyr 1101.

It is possible that the phosphorylation observed on Tyr 845 and/or 1101 on the receptor could contribute to increased tumorigenic potential. Results in section III demonstrated that cells expressing HER1 bearing a Tyr to Phe mutation at position 845 are defective in their ability to synthesize DNA in response to treatment with EGF or serum. Moreover, cells expressing wild type HER1 and kinase defective c-Src (EGFR/K- cells) are impaired in their ability to form colonies in soft agar, a measure of transformation [Tice et al., 1998]. Tyr 845 is not phosphorylated in these cells, as revealed by phosphotryptic mapping, which further argues for the importance of this phosphorylation in HER1-mediated transformation. Thus, phosphorylation of this tyrosyl residue, potentially resulting in hyperactivation of the receptor and increased phosphorylation of downstream substrates, could be a mechanism responsible for potentiation of tumorigenesis. Studies are currently underway to determine the properties of anchorage-independent growth, and tumorigenicity,of 10T845 cells.

Surprisingly, cells expressing the Y845F variant HER1 are not impaired in their ability to activate substrates downstream of HER1: no apparent differences in the phosphorylation and/or activation of a number of HER1 effectors have been found in either the 10T845 cells and the EGFR/K- cells. Moreover, our preliminary evidence suggests that the Y845F HER1 is not impaired in its catalytic activity. There are two possible explanations for this: 1. Another substrate or signalling pathway is affected, which we hope to reveal in our continuing investigations: 2. Compensatory mechanisms exist so that at a biochemical level, the receptor appears to function normally. Such compensatory mechanisms might include increases in phosphorylation on other sites on HER1, and this possibility will be investigated via tryptic mapping. In either case, the dramatic biological responses (impaired EGF- induced DNA synthesis and growth in soft agar) seen resulting from cells expressing the Y845F HER1 variant indicate that this receptor is able to act in a dominant negative fashion.

Interestingly, the c-Src mediated phosphorylation of Tyr 845 may reflect a more general mechanism of receptor function. While EGF treatment is known to drive the association between c-Src and HER1 and to trigger the phosphorylation at position 845, other agonists, which signal through GPCRs or cytokine receptors, have a similar effect. Evidence in the literature supports the idea that HER1 can act downstream of a variety of receptor types, and its kinase activity may be necessary for agonists (such as GPCR agonists) to signal to HER1 effectors: for example, Daub et al. [1996, 1997] showed that expression of a kinase inactive HER1 abrogated the increase in MAPK activity in response to LPA. Our findings suggest that the c-Src mediated phosphorylation of Tyr 845 is also a crucial event in this signalling cascade, since fibroblasts expressing the Y845F variant HER1 are impaired in their ability to synthesize DNA in response to serum, the active ingredient in which is LPA. The fact that Tyr 845 is phosphorylated in response to LPA as well as ET, another GPCR agonist, and GH, a cytokine receptor ligand, suggests that this phosphorylation may not be restricted to EGF treatment. Thus, this phosphorylation could allow optimal HER1 function in response to a variety of stimuli that can act via traditional EGF- mediated pathways.

Progess with regards to Statement of Work:

Aim I: Testing the model of synergistic interactions between c-Src and HER1 in human breast tumor tissues and cell lines.

A. Extend the screening of a panel of human breast tumor tissues and cell lines for expression levels of c-Src and HER1, determine under which conditions they physically associate, whether novel sites of tyrosyl phosphorylations are present on the c-Src associated receptor, and whether tyrosyl phosphorylation of HER1 substrates is increased in cell lines expressing high levels of HER1 and c-Src.

The experiments outlined above have been completed, and are the subject of a recent publication [Biscardi et al., 1998]. The exception concerns the investigation of human breast tumor tissue samples. The majority of the samples I obtained came from earlier stage, in situ tumors, which are not known to overexpress HER1 [Sainsbury et al., 1987; Toi et al., 1991; Battaglia et al., 1988]. Thus, while 20 samples were screened, only one of these was positive for both HER1 overexpression and c-Src association. Obtaining samples from later stage, more aggressive tumors would be difficult, since patients with these types of tumors are usually not good candidates for surgery. For this reason, the studies in Aim I focussed on tumor cell lines instead.

B. Determine whether the level of expression of HER1 and/or c-Src correlates with the transforming potential of human tumor cell lines.

During my investigations I found that the c-Src/HER1 heterocomplex occurred in all cell lines where HER1 was overexpressed, even when c-Src was only moderately expressed (eg, MDA231 cells). The studies in nude mice corroborate the synergistic effects of mutual overexpression of both c-Src and HER1 in breast cancer cell lines.

C. Create breast tumor cell lines which overexpress both c-Src and HER1, and examine them for enhanced tumorigenicity, complex formation, and novel sites of phosphorylation.

These studies were performed in the MDA468 cell line, as indicated above. Overexpression of c-Src in MDA468 cells did enhance phosphorylation of HER1 at position 845, as well as increase tyrosyl phosphorylation of Shc. However, these cells caused only a modest increase in tumor size in nude mice, with respect to parental MDA468 cells. (data not shown). Studies involving DNA synthesis and anchorage independent growth have not yet been performed, since I have chosen to focus on characterizing the intriguing Tyr 845 phosphorylation at the present time.

D. Attempt to inhibit growth by introducing blocking peptides or domains of HER1 in cell lines exhibiting c-Src/HER1 enhanced transforming potential.

These studies will be undertaken in the second year of the grant proposal.

Aim II: Structure/function analysis of HER1/c-Src interaction in the 10T1/2 system.

A. Determine regions of HER1 required for enhanced tumorigenicity: examination of juxtamembrane, kinase, and C terminal domains.

These studies have been temporarily put on hold, in order to pursue subaims B and C, below.

B. Determine the physiological relevance of the kinase domain to increased tumorigenic potential.

The importance of Tyr 845 phosphorylation has been described above, in section II of the Results and Discussion.

B. Determine the physiological relevance of the HER1 C terminus to increased tumorigenic potential.

I have just begun investigating a set of NIH3T3 cell lines which overexpress HER1 bearing point mutations at each of the C terminal phosphorylation sites, with regards to a potential site of c-Src interaction. Unfortunately, these cell lines all express widely different amounts of HER1 , which clouds interpretation of the results. I will create new cell lines which express equivalent amounts of receptor either by subcloning the existing cell lines, or by re-transfection if necessary.

Aim III: Interdiction of the HER1/c-Src signalling pathways in the 10T1/2 model system and in breast cancer cell lines.

This Aim will be undertaken during years 2 and 3 of the grant proposal.

Conclusions:

1. The synergistic interactions between c-Src and HER1, which were first described in a mouse fibroblast model system, also hold true in human breast cancer: In cell lines where HER1 was overexpressed, c-Src and HER1 physically associate, Shc phosphorylation and MAPK activity are increased with respect to cell lines which do not overexpress HER1, and larger tumors are formed in nude mice.
2. I have identified two novel sites of tyrosyl phosphorylation on the c-Src associated HER1, namely Tyr 845 and Tyr 1101. While it is clear that phosphorylation of tyrosines analogous to Tyr 845 in other tyrosine kinases is critical for their function, this phosphorylation has been difficult to detect. My studies have suggested that this phosphorylation is very labile, and is dependent on the presence of a catalytically active c-Src. Since expression of kinase deficient c-Src abrogates Tyr 845 phosphorylation, it is possible that c-Src directly phosphorylates this site on HER1.
3. Phosphorylation of HER Tyr 845 is critical for HER1 mediated DNA synthesis, as demonstrated by studies investigating BrDU uptake in cells expressing a Y845F mutant form of HER1. The Y845F mutant decreases BrDU uptake to basal levels in response to both EGF and serum, indicating that phosphorylation at this site is an important step in pathways emanating from both EGF and LPA.
4. A number of biological agonists which act through either GPCR (LPA, endothelin) or a cytokine receptor (GH) also cause association between c-Src and HER1 and phosphorylation of Tyr 845. In light of recent data which shows that "cross talk" exists between HER1 pathways and pathways

involving GPCR and cytokine receptors, phosphorylation of Tyr 845 may be a general mechanism that allows the receptor to function in response to a variety of stimuli.

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FIGURE LEGENDS

Fig. 1. Expression levels of HER1and c-Src in a panel of human breast tumor cell lines. Protein extract antibody (Q9), or a nonspecific control antibody (C). Control immunoprecipitations (Panel A, lane 4; Panel B, lane 5) were prepared from cells stimulated for 10 min with EGF. Immunocomplexes were then washed and subjected to an *in vitro* kinase reaction using γ-[^{32}P]-ATP. Labeled proteins were resolved by SDS-PAGE and visualized by autoradiography. Background bands in the c-Src region in the control antibody lane of Panel B are of unknown origin. c-Src migrates above them, as indicated. Within each cell line, stimulated or unstimulated, equal amounts of c-Src protein were immunoprecipitated, as determined by immunoblotting with the 2-17 c-Src specific antibody (not shown). This experiment was repeated 5 times with nearly identical results. Other cell lines were also analyzed in the same manner. All results are summarized in Table I.

Fig. 2. *In vivo* association between HER1and c-Src in 10T½ cells overexpressing c-Src and HER1 (5HR11 cells, **Panel A**) or in human breast cancer cell lines (**Panel B**). Equal amounts (500μg) cell lysate (in CHAPS detergent) prepared from nonstimulated cells or cells stimulated with 100 ng/ml EGF for the indicated times in Panel A or for 10 min in Panel B were immunoprecipitated with either a c-Src specific antibody (Q9), or a nonspecific control antibody (C). Control immunoprecipitations (Panel A, lane 4; Panel B, lane 5) were prepared from cells stimulated for 10 min with EGF. Immunocomplexes were then washed and subjected to an *in vitro* kinase reaction using γ-[^{32}P]-ATP. Labeled proteins were resolved by SDS-PAGE and visualized by autoradiography. Background bands in the c-Src region in the control antibody lane of Panel B are of unknown origin. c-Src migrates above them, as indicated. Within each cell line, stimulated or unstimulated, equal amounts of c-Src protein were immunoprecipitated, as determined by immunoblotting with the 2-17 c-Src specific antibody (not shown). This experiment was repeated 5 times with nearly identical results. Other cell lines were also analyzed in the same manner. All results are summarized in Table I.

Fig. 3. **Panel (A)** Expression levels of HER1 and c-Src in a panel of human breast tumor tissues and normal breast tissue (nml). Protein extracts (80 μg) from the various tissues were separated on SDS-PAGE, transfererred to nitrocellulose, immunoblotted with HER1 specific antibody 3A/4A (top) or c-Src antibody 2-17 (bottom), followed by detection with [^{125}I] anti-mouse IgG. **Panel (B)** *In vivo* association between HER1 and c-Src in human breast tumor tissue. Tissue samples were homogenized in CHAPS detergent, and equal amounts of lysate (500 μg) were immunoprecipitated with either c-Src antibody Q9 or a nonspecific antibody. Immunocomplexes were washed and subjected to an *in vitro* kinase reaction using γ-[^{32}P]-ATP. Labeled proteins were resolved by SDS-PAGE and visualized by autoradiography.

Fig. 4. Specific activity of c-Src in 10T½ murine fibroblasts and human breast tumor cell lines. c-Src was immunoprecipitated from 500 μg RIPA cell lysate using a pool of 2-17 and GD11 Mabs. 2 μg acid-treated enolase and 10 μCi γ-[^{32}P]-ATP were added to RIPA-washed immunoprecipitates, and kinase reactions were carried out in a PIPES/$MnCl_2$ buffer as described in Materials and Methods. Labelled proteins were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to autoradiography. c-Src, immunoprecipitated from an equal amount of lysate from each cell line, was immunoblotted with 2-17 antibody and detected with [^{125}I]-rabbit anti-mouse IgG. The amount of radiolabel incorporated into enolase and the amount of c-Src in the precipitates were quantitated by densitometry, and the specific activities of c-Src were calculated by dividing the densitometric level of enolase phosphorylation by the amount of c-Src immunoprecipitated from each cell line. With the exception of Neo cells, results shown are the averages of 3-5 independent experiments per cell line.

Fig. 5. Elevated levels of Shc tyrosyl phosphorylation in breast tumor cell lines overexpressing HER1 and c-Src. Lysates (500 μg) were prepared from nonstimulated MDA468, MDA231, BT-549, MCF7 or ZR75 cells or cells stimulated with 100 ng/ml EGF for 10 min, and extracts were immunoprecipitated with either Shc antibody or a nonspecific control antibody and immunoblotted with either pTyr antibody **(Panel A)** or Shc antibody (Panel B), using ECL as a detection method. This experiment represents one of 5, all of which yielded similar results.

Fig. 6. Activation of MAPK in breast tumor cell lines overexpressing HER1 and c-Src. Proteins in 75 μg lysate from the indicated cell lines, each of which had been treated with or without 100 ng/ml EGF for 2 min, were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with either phospho-MAPK antibody or pan-MAPK antibody (B3B9). This experiment was repeated 3 times with nearly identical results.

Fig. 7. Phosphotryptic peptides radiolabeled *in vitro* or *in vivo*. For *in vitro* labeling (Panels A and B), 5HR and NeoR cells were stimulated with 100 ng/ml EGF for 30 min, followed by lysis in CHAPS buffer and immunoprecipitation of extract proteins with either c-Src- specific (GD11) or EGFR- specific (3A/4A) antibody. Precipitated proteins were then subjected to an *in vitro* kinase reaction. For *in vivo* experiments (Panel C), cells were labeled for 18 hr in phosphate free media containing [^{32}P]-orthophosphate, stimulated with 100 ng/ml EGF for 5 min in the presence of pervanadate, and lysed in CHAPS buffer. Extracts were immunoprecipitated with GD11 antibody. c-Src-associated, *in vivo*- labeled EGFR was eluted from gel slices, and samples were trypsinized and processed as previously described (17), using isobutyric acid buffer to separate peptides in the second dimension. Labeled peptides were visualized by autoradiography. Panel A: *In vitro* labeled EGFR immunocomplexes from NeoR cells (2000 cpm); Panel B: *In vitro* labeled, c-Src- associated EGFR from 5HR cells (2000 cpm); Panel C: c-Src- associated EGFR from 5HR cells labeled *in vivo* (3000 cpm).

Fig. 8. Phosphorylation of peptides "0" and "3" in metabolically labeled, pervanadate treated cells. NeoR and 5HR cells were incubated for 18 hr with [^{32}P]-orthophosphate as above. Pervanadate (3 mM H_2O_2 and

5 μM Na_3VO_4) was added (Panels B and D) or not (Panels A and C) along with 100 ng/ml EGF for 5 min prior to lysis in RIPA detergent buffer. EGFR was immunoprecipitated with Mabs 3A/4A, and the receptor processed for phosphotryptic analysis as described above. Panel A: EGFR from NeoR cells; Panel B: EGFR from pervanadate- treated NeoR cells; Panel C: EGFR from 5HR cells; Panel D: EGFR from pervanadate- treated 5HR cells. ~3000 cpm were loaded per TLC plate.

Fig. 9. Edman degradation of peptides "0" and "3". Peptides "0" and "3" were isolated by HPLC and subjected to automated Edman analysis. (A) ^{32}P from peptide "0" was released at the second cycle, indicating a phosphorylated tyrosine at position 2; (B), ^{32}P from peptide "3" was released at the fourth cycle, indicating a phosphorylated tyrosine at position 4.

Fig. 10. Identification of Peptide "0". The octapeptide E(Y-P)HAEGGK was synthesized to contain phosphorylated Tyr 845 and analyzed by 2D electrophoresis/chromatography on TLC plates, either alone (Panel A) or in a mixture with total *in vitro*- labeled tryptic phosphopeptides derived from the receptor which co-precipitated with c-Src (Panel C). The synthetic phosphopeptide, detected by hypochlorite spraying, co-migrated with tryptic peptide "0", verifying Tyr 845 as the site on the receptor whose phosphorylation is dependent on c-Src. Panel B, total phosphopeptides from c-Src-associated receptor alone. Panel D, sequence homology between the peptide containing Tyr 416 of c-Src and the peptide containing Tyr 845 of the EGFR.

Fig. 11. Identification of peptide "3". *In vitro*- phosphorylated peptide "3" (as in Fig. 5B) was scraped and eluted from the TLC plate and subjected to digestion with proline-directed protease. Undigested or digested, eluted peptide "3" was then analyzed by 2D TLC either alone (Panels A and B, respectively) or mixed (Panel C). The altered mobility of digested peptide "3" indicates the presence of a proline in the sequence and identifies the peptide as containing Tyr 1101.

Fig. 12. Phosphorylation of Tyr 845 and 1101 in MDA468 breast tumor cells. MDA468 or MDA468c-Src cells were stimulated with 100 ng/ml EGF for 30 min, followed by lysis in CHAPS buffer and immunoprecipitation of extract proteins with either c-Src- specific (GD11) or EGFR- specific (F4) antibody. Precipitated proteins were then subjected to an *in vitro* kinase reaction. The labeled EGFR was eluted from gel slices, and samples were trypsinized and processed as previously described in the legend to Fig. 3. Labeled peptides were visualized by autoradiography. Panel A: Phosphotryptic peptides from *in vitro* labeled EGFR immunocomplexes from MDA468 cells (4000 cpm). Panel B: Protein extracts (50 μg) from MDA468 parental, 5HR, or MDA468c-Src cells which overexpress c-Src, were separated by SDS-PAGE and subjected to immunoblotting with GD11 antibody. Panel C: Phosphotryptic peptides from *in vitro* labeled, c-Src- associated EGFR from MDA468c-Src cells (4000 cpm); Panel D: MDA468 and MDA468c-Src cells were lysed in RIPA buffer and stimulated with EGF for the indicated times. 500 μg protein extract was immunoprecipitated with Shc antibody, followed by immunoblotting with either pTyr antibody (top) or Shc antibody (bottom). Primary antibody binding was detected with the Enhanced

Chemiluminescence (Amersham, Buckinghamshire, England) method. The data shown are representative of 3 independent experiments.

Fig. 13. Y845 is not phosphorylated in HER1 complexed with kinase deficient c-Src. Panels A-D: The 170 kDa bands that were phosphorylated *in vitro* in c-Src (panels B-D) or receptor (panel A) immunecomplexes prepared from the indicated cell lines were excised, digested with trypsin, resolved by 2-D electrophoresis/chromatography, and subjected to autoradiography. The positions of peptides containing Y845 and Y1101, which were identified previously [Biscardi et al., 1998b] are indicated. Panels E and F: receptor immunoprecipitates from the indicated cell lines that had been metabolically labelled with $^{32}P_i$ were analyzed as in panels A-D. Equal cpms were loaded in panels A-D and in panels E and F.

Fig. 14. Phosphorylation of Y845 is essential for HER1 function. 5H cells were transfected with plasmid DNA encoding Y845F or wild type HER1, cultured for 2 days, serum starved for 30 hr, and left untreated or treated with either 40 ng/ml EGF or 10% serum for 18 hr. 100 μM BrDU was added to all cells at the time of mitogen addition. Cells were fixed and co-stained for HER1 expression and BrDU incorporation. 35-75 cells were analyzed for each variable in 3 independent experiments.

Fig. 15. Multiple agonists induce HER1 phosphorylation and association with c-Src. 5HR cells were treated for 10 min with EGF (100 ng/ml), endothelin (10 μM), growth hormone (500 ng/ml), isoproterenol (10 μM), lysophophatidic acid (10 μM) or thrombin (2U/ml), lysed in CHAPS buffer, and subjected to either HER1 immuoprecipitations with Mab F4 (Panel A) or c-Src immunocomplex kinase assays with Mab GD11 (panel B). In panel A, phosphorylated HER1 was visualized by immunoblotting with Ptyr antibody RC20 and ECL. The c-Src associated HER1 in panel B was visualized by autoradiography.

Fig. 16. Endothelin, growth hormone, LPA, and EGF all induce phosphorylation of Tyr 845 of the c-Src associated HER1. 5HR or EGFR/K- cells were processed for *in vitro* immunocomplex kinase reactions as described in Fig. 7, and the c-Src associated HER1 was excised and digested with trypsin. Panels A-D: c-Src associated HER1 derived from 5HR cells; panels E-F: c-Src associated HER1 derived from EGFR/K- cells.

Table I: Relative Levels of HER1 and c-Src in Human Breast Carcinoma Cell Lines

Cell line	HER1	c-Src	ER	c-Src/HER1 Association
MDA-MB175	*	9.9	++	-
UACC-893	*	*	-	ND
UACC-812	*	*	-	-
SK-BR-3	12.6	19.4	+	+
MDA-MB361	*	37.4	+	-
MDA-MB453	*	*	-	-
MDA-MB468	39.5	4.9	-	+
ZR75-B	*	6.2	++	-
BT-474	*	*	+	-
BT-549	6.9	13.4	-	+
MDA-MB231	7.6	2.9	-	+
BT-20	3.4	13.5	-	+
MCF-7	1	6.4	++	-
MDA-MB415	*	7.6	ND	-
Hs578Bst[1]	1	1	+	-

[1]normal human breast epithelial cells used as negative control

(*) = below limits of detection

ND=not determined; ER= estrogen receptor, whose relative levels were determined by immunoblotting. Relative levels of HER1 and c-Src were determined by densitometry. The lowest detectable signal, in Hs578Bst cells, was arbitrarily assigned a value of 1, and all other values were expressed relative to this signal. The immunoblot shown in Fig. 1 is a representative of several experiments and was chosen in an effort to show the varying levels of signals in all the cell lines.

Table II: Tumor Formation in Nude Mice

	day 28:		day 50:	
Cell Line	%Tumors	Size (mm^3)	%Tumors	Size (mm^3)
NeoR1	11	<1	33	<1
5HR11	100	290	*	*
MDA 468	80	14	90	16
MDA 231	57	12	100	10
BT-549	10	1	10	1
ZR75-1	67	6	67	13[†]
MCF7	11	<1	22	1

For each cell line, n=4-5 mice, 2 subcutaneous injection sites per mouse, $2x10^6$ cells/site. "*" indicates mice were sacrificed after 35 days due to large size of tumors. Tumor size was measured with calipers, and volume was calculated as described in Materials and Methods.
[†]In mice injected with ZR75-1 cells, a single site developed an aberrantly large tumor. This data point was not considered in the calculations above.

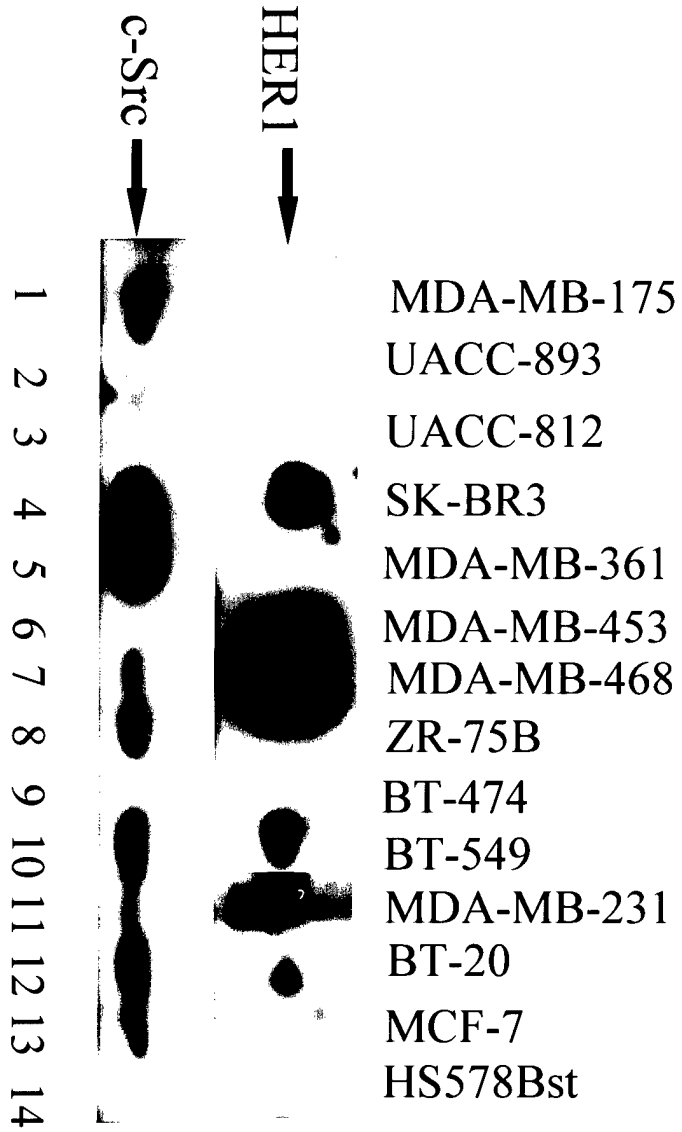
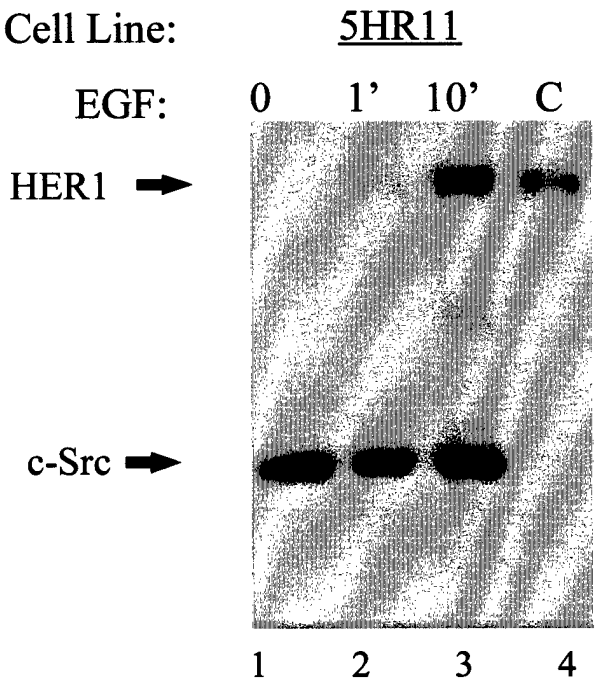

c-Src
HER1
1 2 3 4 5 6 7 8 9 10 11 12 13 14
MDA-MB-175
UACC-893
UACC-812
SK-BR3
MDA-MB-361
MDA-MB-453
MDA-MB-468
ZR-75B
BT-474
BT-549
MDA-MB-231
BT-20
MCF-7
HS578Bst

Figure 1

A


Cell Line: 5HR11
EGF: 0 1' 10' C
HER1
c-Src
1 2 3 4

B

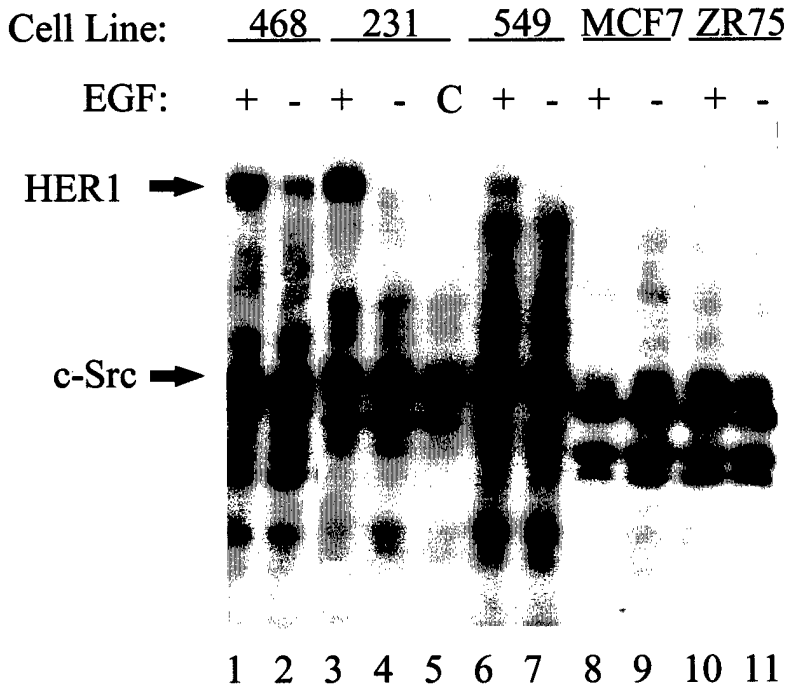
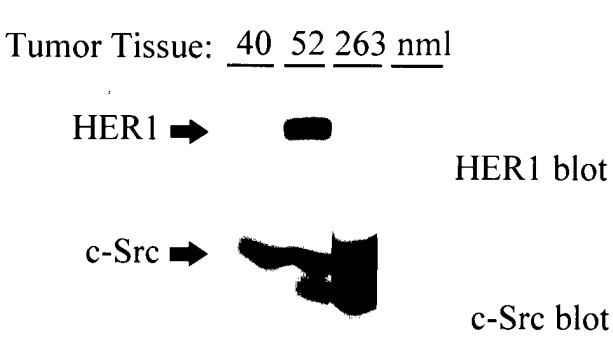

Cell Line: 468 231 549 MCF7 ZR75
EGF: + - + - C + - + - + -
HER1
c-Src
1 2 3 4 5 6 7 8 9 10 11

Figure 2

In vivo association between c-Src and HER1 in human breast tumor samples

A



B

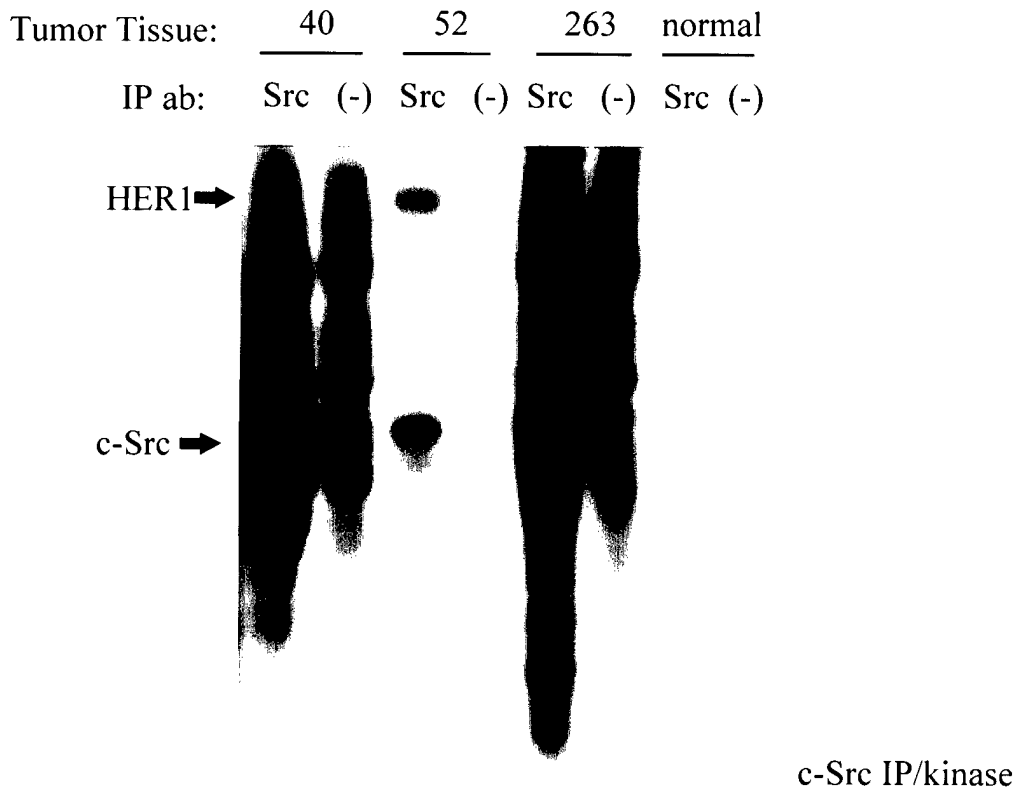


Figure 3

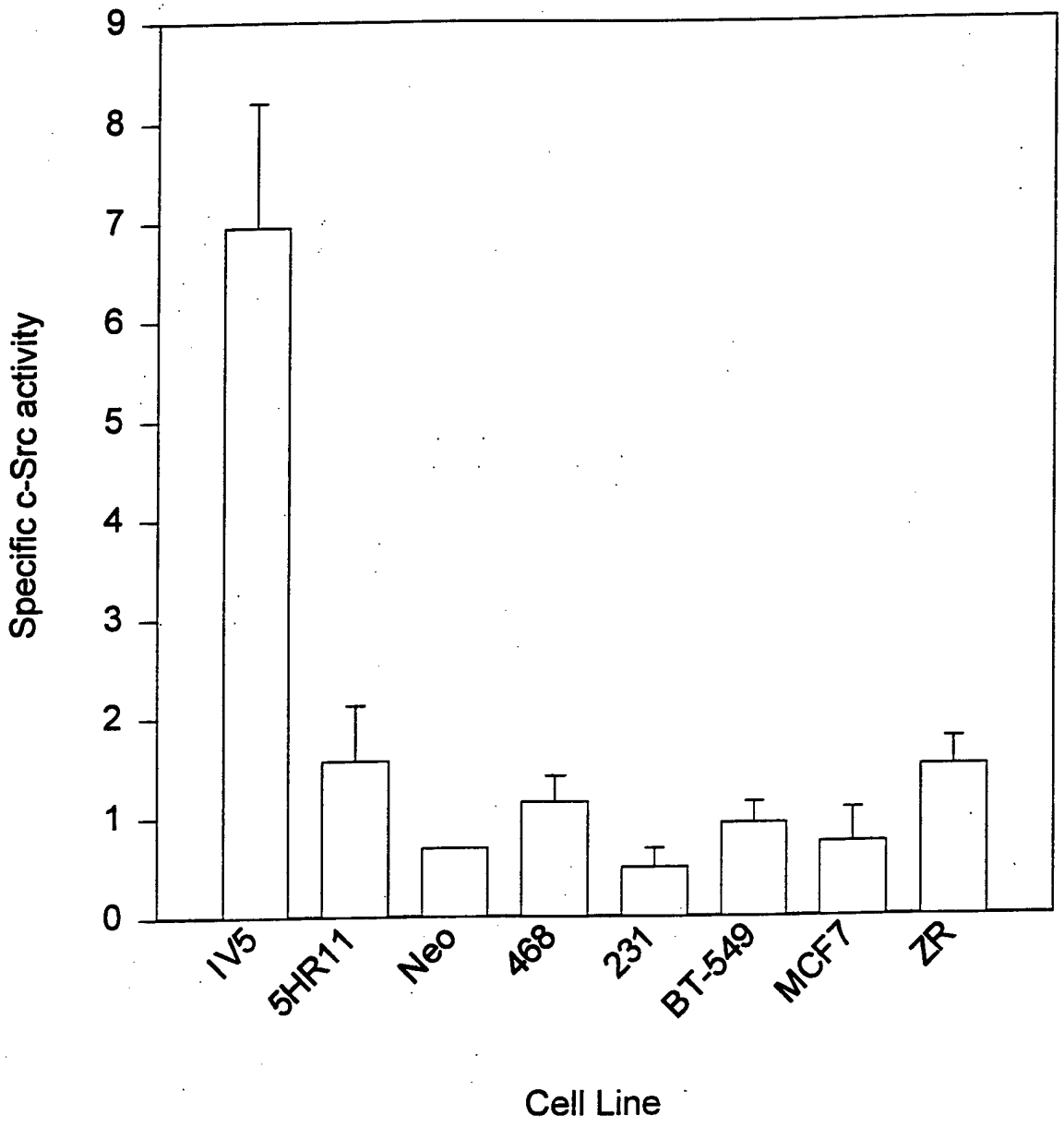

c-Src specific activity in breast cancer cell lines
Specific c-Src activity
9
8
7
6
5
4
3
2
1
0
IV5
5HR11
Neo
468
231
BT-549
MCF7
ZR
Cell Line

Figure 4

Phosphorylation of Shc in human breast cancer cell lines

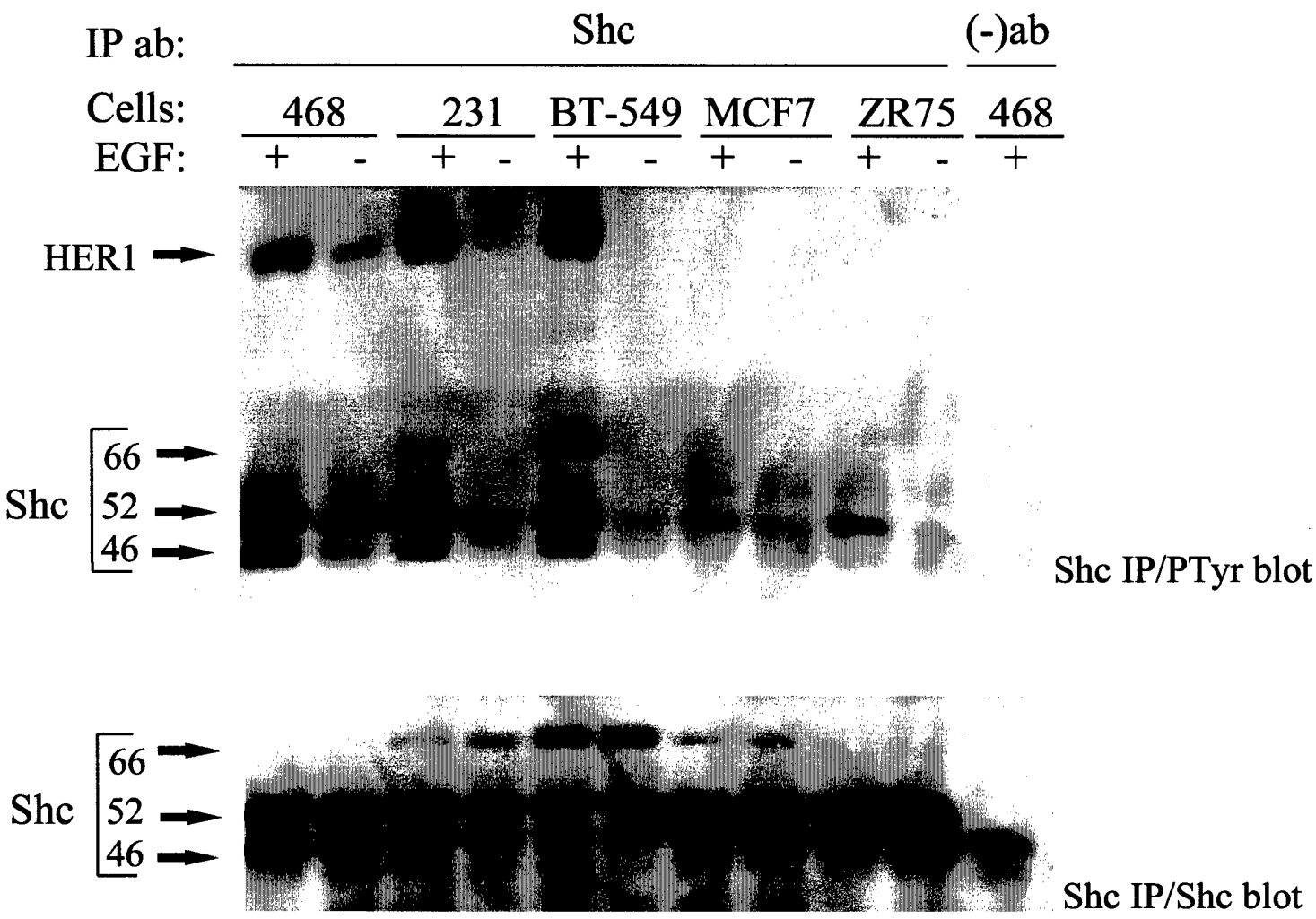


Figure 5

Activation of MAPK in human breast cancer cell lines

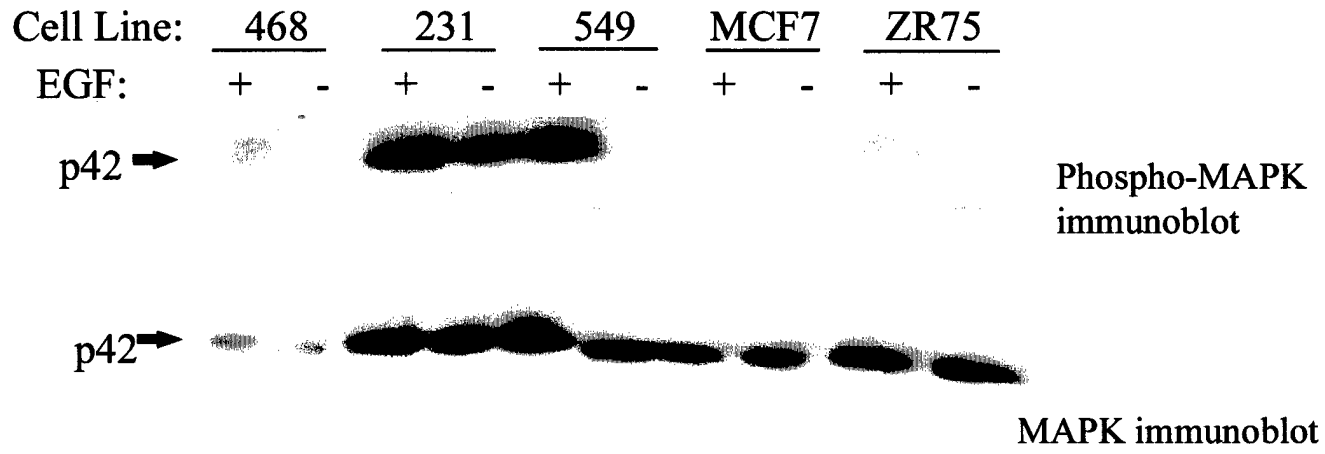
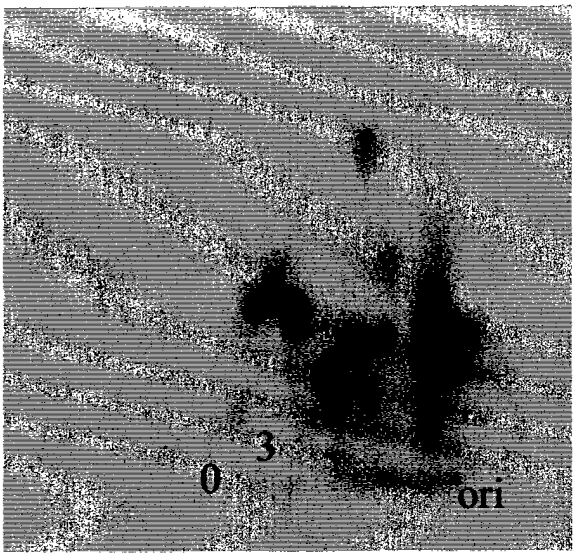


Figure 6

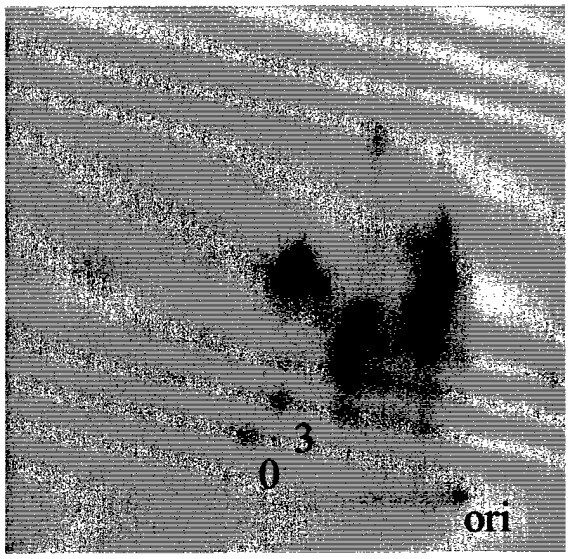
A. NeoR R *in vitro*
ori
B. 5HR Src/R *in vitro*
3
0
ori
C. 5HR Src/R *in vivo*+ PV
ori

Figure 7

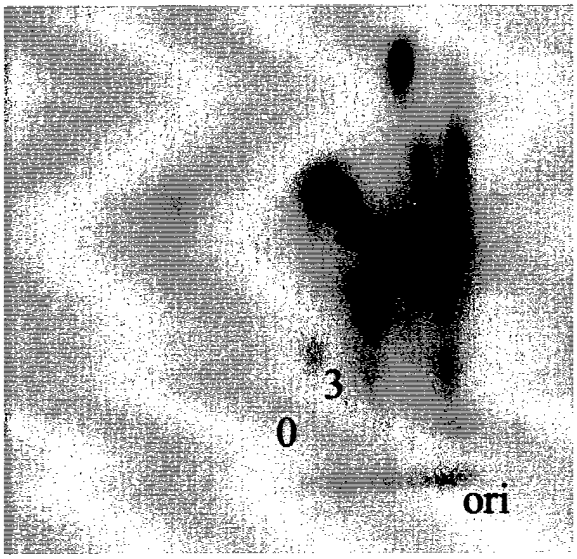
A. NeoR cells/R IP (-) PV



B. NeoR cells/R IP +PV



C. 5HR cells/R IP (-) PV



D. 5HR cells/R IP + PV

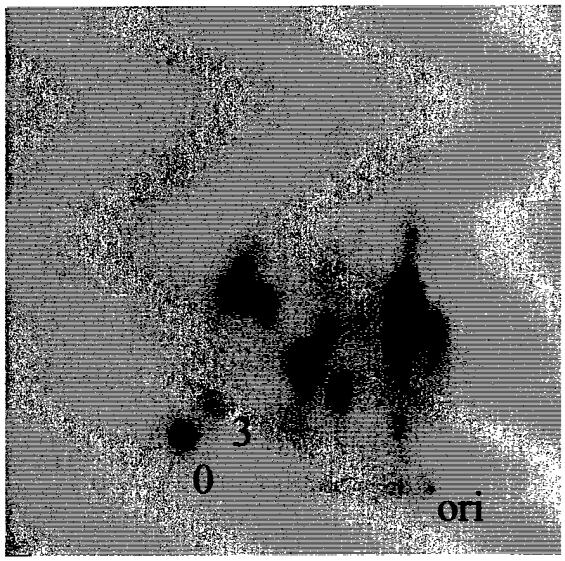
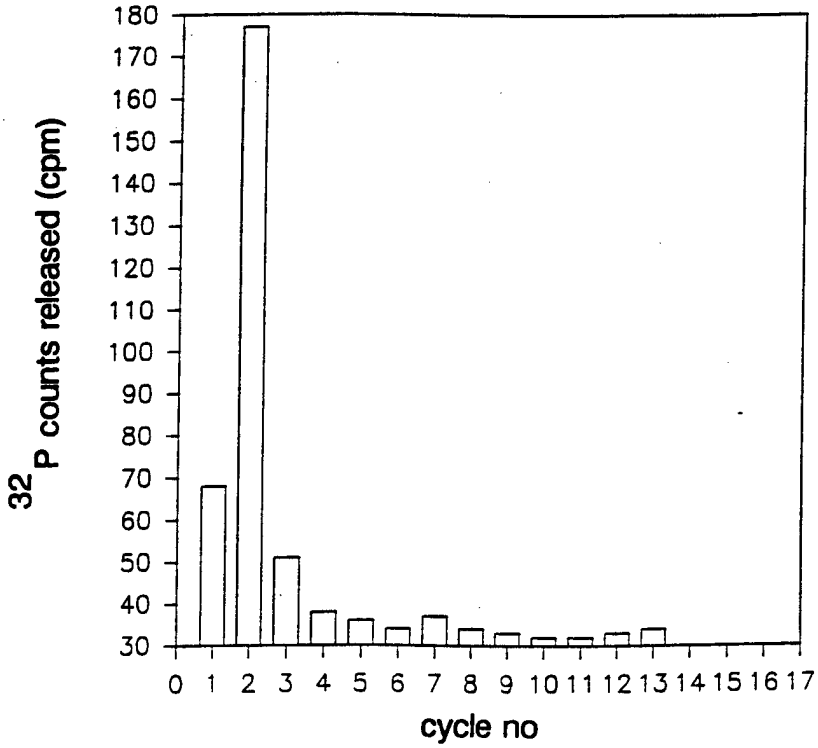


Figure 8

A) peptide "0"



B) peptide "3"

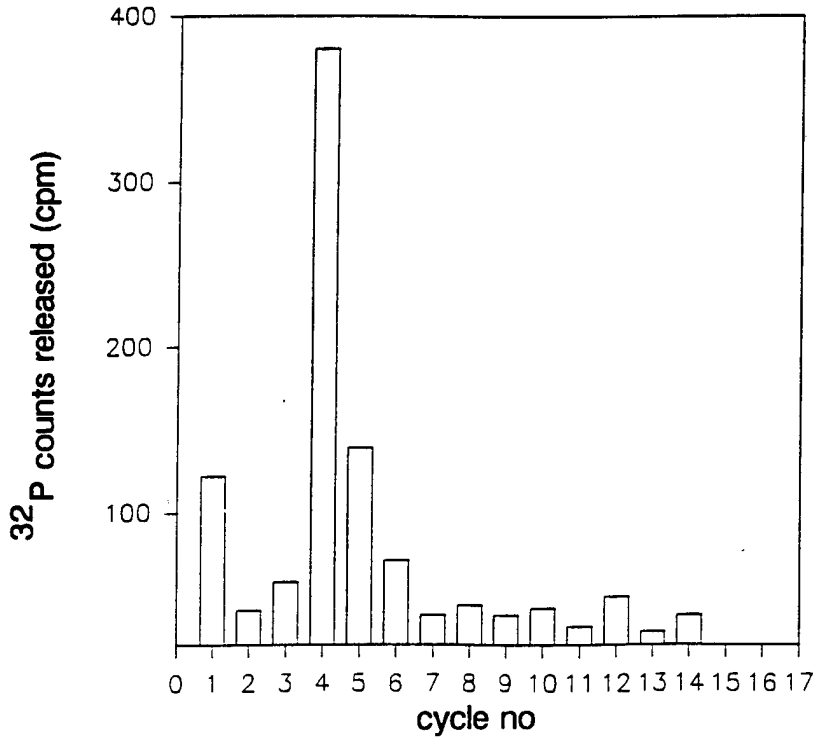
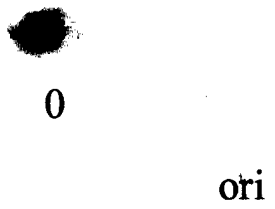


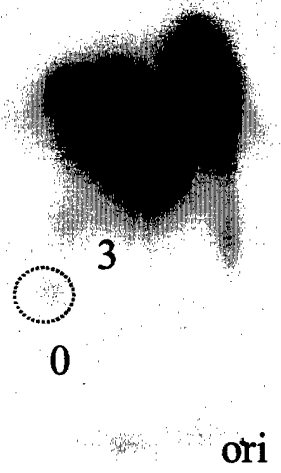
Figure 9

A. peptide alone



B. Src/R alone

C. In vitro Src/R + peptide



D

Src: glu **tyr** thr ala arg gln gly ala
416

EGFR: glu **tyr** his ala glu gly gly lys
845

Figure 10

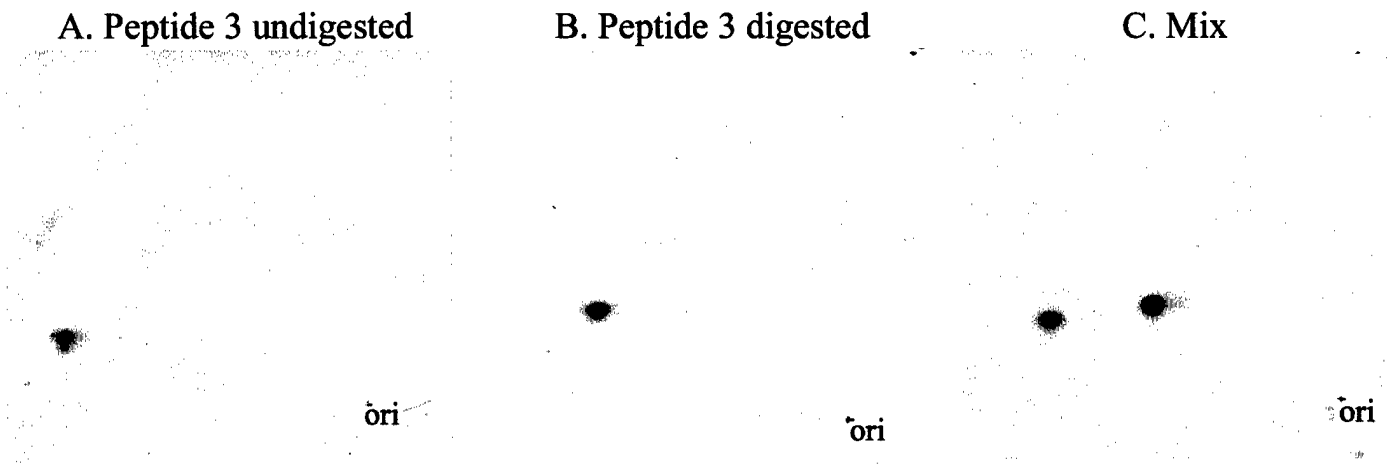
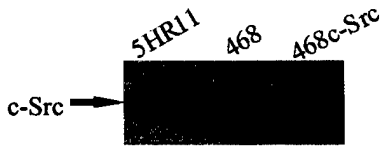


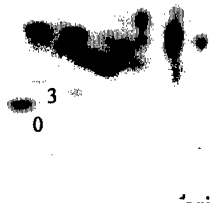
Figure 11

A. 468 Src/R *in vitro*

B. c-Src immunoblot



C. 468c-SrcOX Src/R *in vitro*



D. Shc IP

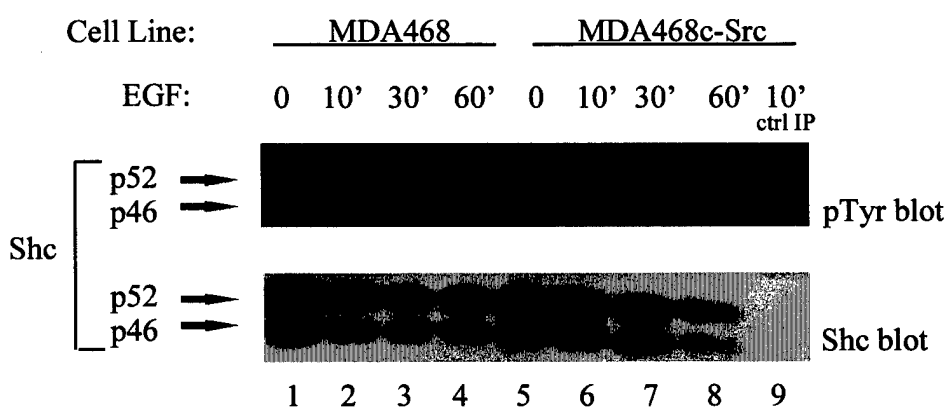


Figure 12

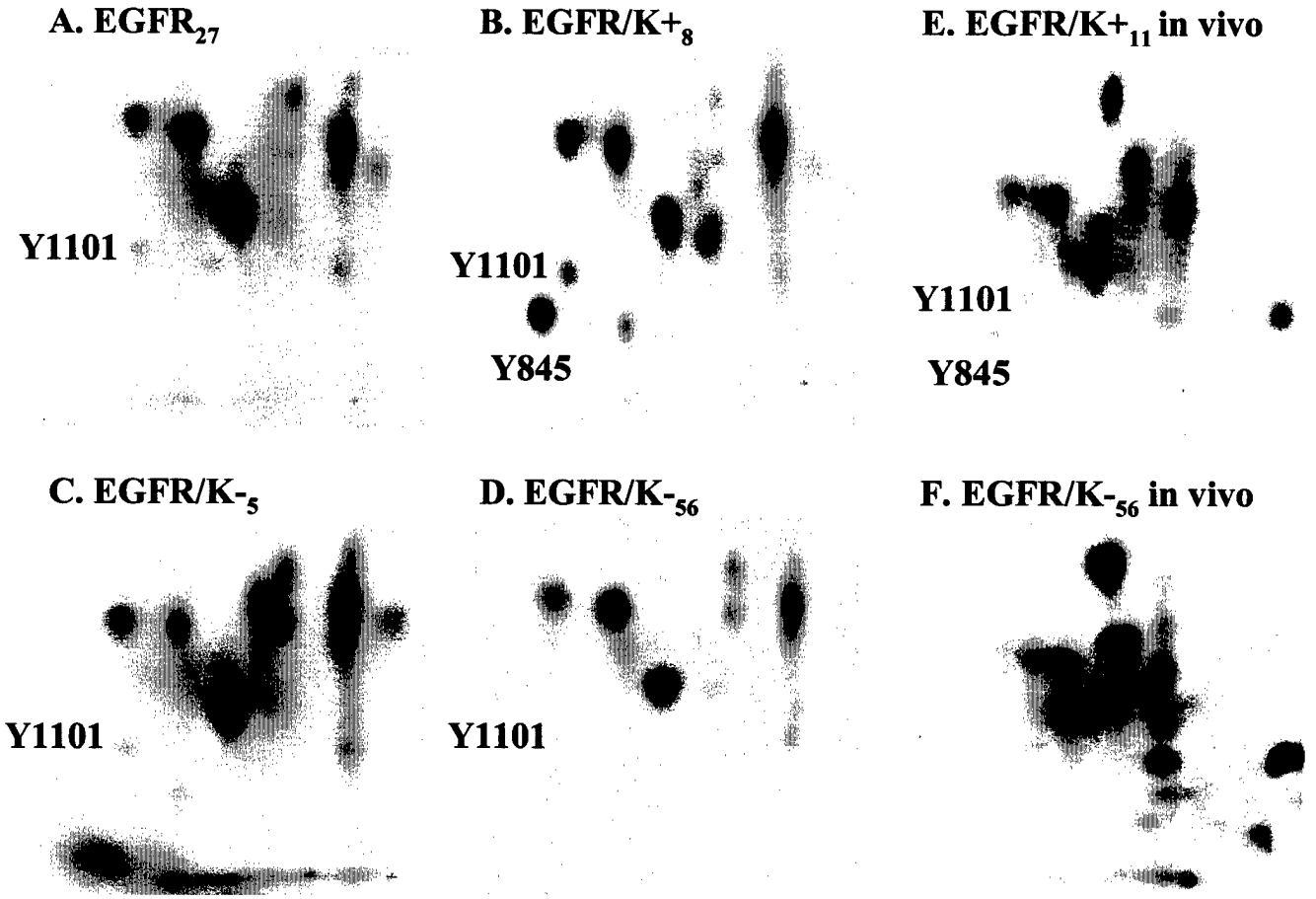

A. EGFR27
Y1101
B. EGFR/K+8
Y1101
Y845
E. EGFR/K+11 in vivo
Y1101
Y845
C. EGFR/K-5
Y1101
D. EGFR/K-56
Y1101
F. EGFR/K-56 in vivo

Figure 13

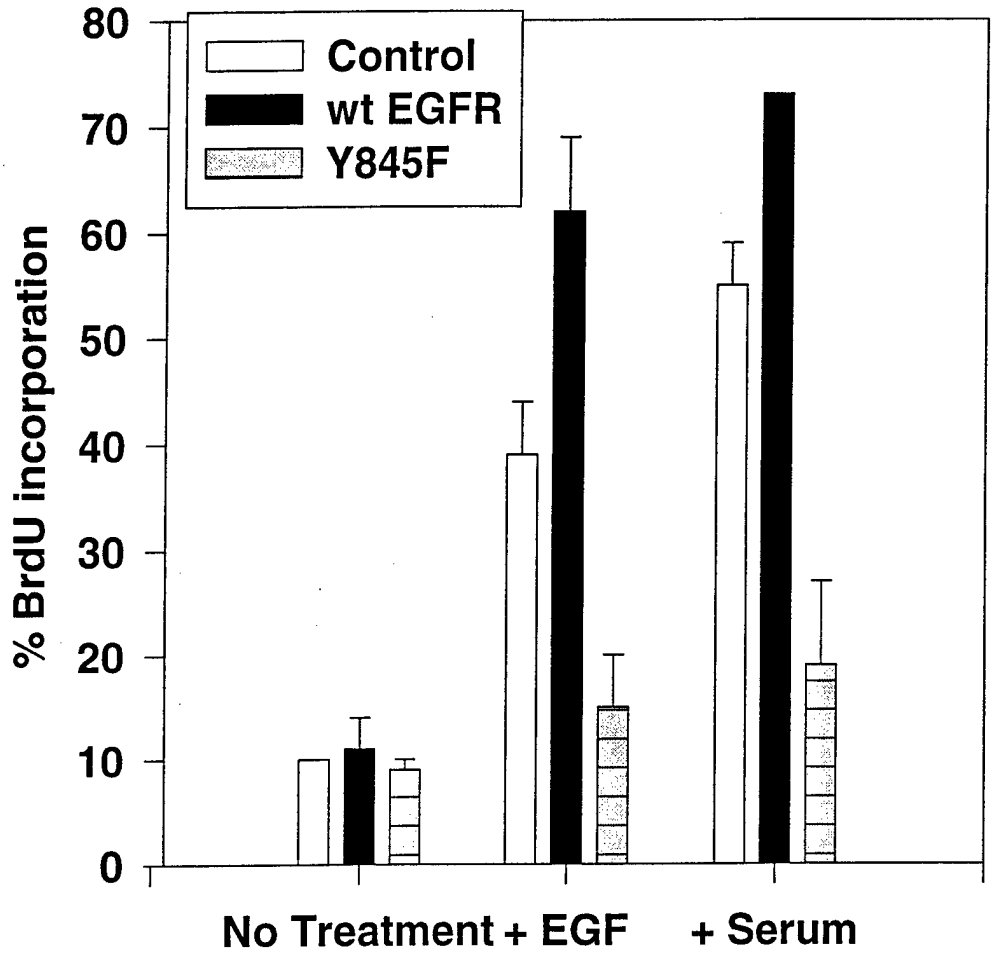

80
70
60
50
40
30
20
10
0
% BrdU incorporation
Control
wt EGFR
Y845F
No Treatment
+ EGF
+ Serum

Figure 14

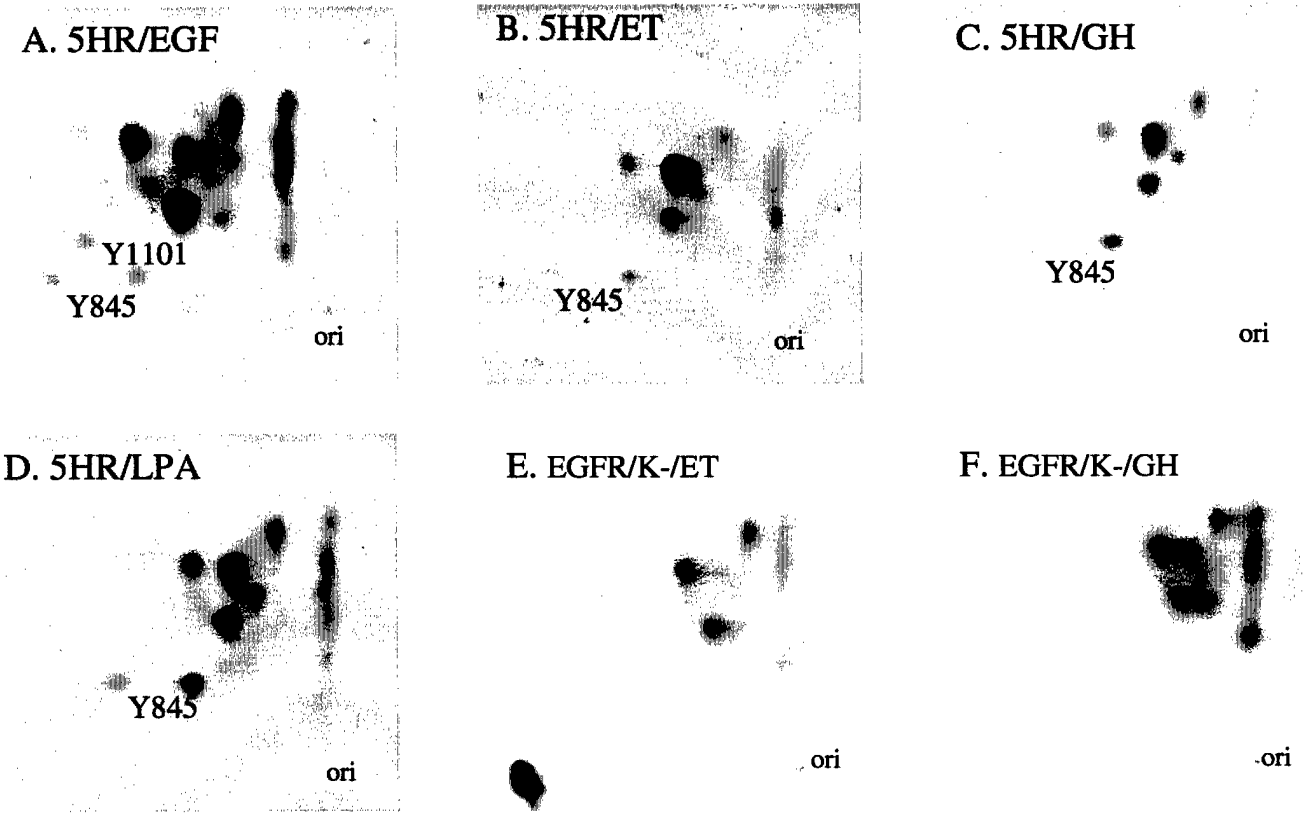

A. 5HR/EGF
Y1101
Y845
ori
B. 5HR/ET
Y845
ori
C. 5HR/GH
Y845
ori
D. 5HR/LPA
Y845
ori
E. EGFR/K-/ET
ori
F. EGFR/K-/GH
ori

Figure 15

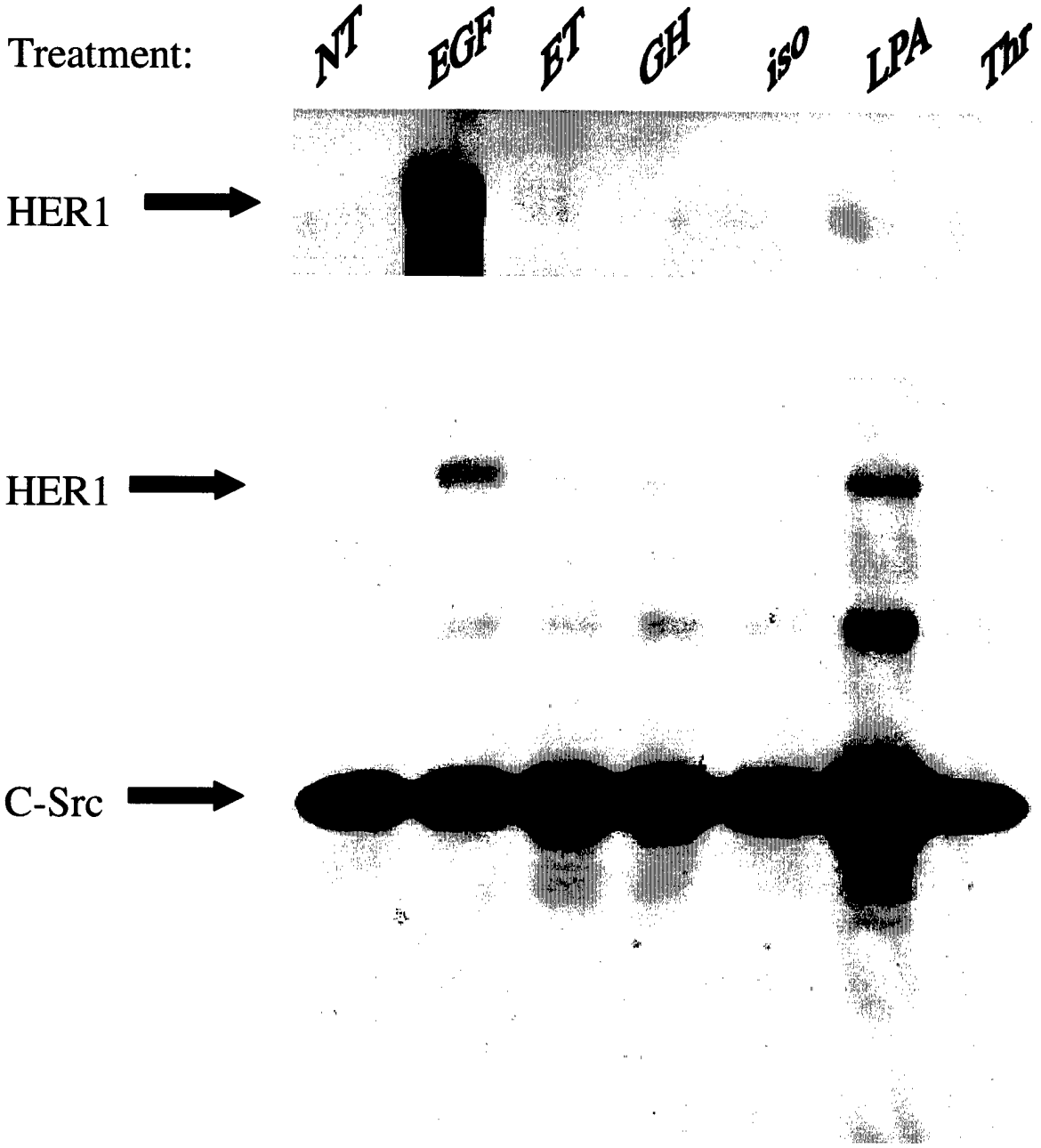

Treatment:
NT
EGF
ET
GH
iso
LPA
Thr
HER1
A. HER1 IP/Ptyr blot
HER1
C-Src
B. C-Src IP/kinase

Figure 16